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COMPARATIVE STUDY OF HOST RESISTANCE OF GUINEA PIGS AND RATS

IV. THE EFFECT OF BACTERIAL FRACTIONS ON THE ADENOSINETRIPHOSPHATASE ACTIVITY OF THE GUINEA PIG AND THE RAT LEUCOCYTE NUCLEI-FREE HOMOGENATES

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The work reported in this paper is part of a general program being conducted by Dr. W. J. Nungester and colleagues in which a search is being made for factors which may be responsible for differences in host resistance to a given microorganism. In this program, studies have been made on guinea pigs and rats in an effort to explain the marked and opposite reaction of these two hosts to *Diplococcus pneumoniae* type I and *Bacillus anthracis*.

This paper is concerned with an investigation of the effect of fractions of *D. pneumoniae* and *B. anthracis* on the enzyme adenosinetriphosphatase (ATPase) of leucocytes obtained from guinea pigs or white rats. The first objective was to partially characterize enzyme ATPase from leucocytes of these hosts. Then, the effects of fractions of pathogens thought to be related to their virulence on this enzyme were studied. In other words, would the enzyme (ATPase) systems of the leucocyte nuclei-free (WBC n.f.) homogenate of a resistant animal differ from those of a susceptible host; this would be an important factor in understanding species resistance of the host to infection.

In this paper evidence is presented that adenosinetriphosphate (ATP) is involved in the metabolism of peritoneal exudates and spleen homogenates. It is also shown that the bacterial fraction known to be associated with virulence will inhibit the enzyme activity of ATPase; the same bacterial fraction activates the ATPase when tissue homogenates are prepared from a host naturally resistant to the parasite.

MATERIALS AND METHODS

Source and preparation of leucocyte nuclei-free (n.f.) homogenates.—White blood cells were obtained for study by inducing exudation into the periotoneal cavities of the donor animals.[1] The day before the cells were to be used 100 ml of saline (0.85 percent NaCl) containing 1 percent glucose were injected intraperitoneally into each animal. The rats were anesthetized to expedite injection but this was not necessary with guinea pigs. The exudate was collected 12 to 18 hours later. The animals were sacrificed by a sharp blow on the head. The exudate was collected from the open abdomen and that from several animals was pooled in a cooled 250 ml centrifuge bottle containing heparin as an anticoagulant. The bottles were kept in a bath of ice water throughout the white blood cell harvest and all centrifugation was done in a room kept at about 2C.

The cells were concentrated by centrifugation at 250 G for 10 minutes. The supernate was discarded and the cells resuspended in isotonic saline. The process was repeated three times. After the last decantation the packed leucocytes were suspended in a minimal quantity of saline

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1. Nungester, W. J. and Ames, A. M. 1948, The relationship between ascorbic acid and phagocytic activity. J. Infect. Dis. **83**: 50–54.

and centrifuged at 500 G for 15 minutes. A 10 percent suspension (using distilled water) was prepared from the packed washed cells. The suspension was frozen and thawed with resulting lysis of the leucocytes. The homogenate thus obtained was centrifuged at 500 G for 15 minutes to separate the nuclei. The slightly opalescent supernatant obtained will be referred to as nuclei-free leucocyte homogenate.

Reagents.—2-amino-2-methyl-1,3-propanediol[8] and 2-amino-2-hydroxy-methyl-1,3-propanediol[7] were kindly supplied by Commercial Solvents Corporation, 17 East 42nd Street, New York 17, New York. 1,3-diaminobutane[7] was generously furnished by Sharples Chemical Inc., Philadelphia 9, Pennsylvania.

Tetrasodium salt of adenosinetriphosphate (ATP) was obtained from the Sigma Chemical Company, St. Louis, Missouri. Analysis showed the material was 95 percent ATP and 5% adenosine diphosphate (ADP) and inorganic phosphate.

Pneumococcal polysaccharides.—Purified pneumococcus type I polysaccharide, SI, was prepared by Dr. F. C. Kull according to the method of Heidelberger, McLeod, Markowitz, and Roe[2] from *D. pneumoniae*, type I Michigan strain no. 76. SI-L4 was prepared from an avirulent strain of *D. pneumoniae*, type I. Professor Alan W. Bernheimer, New York University College of Medicine, graciously supplied the pneumococcus type III polysaccharide, SIII, used in this study.

B. anthracis *antigen.*—Lyophilized anthrax antigen was obtained from Dr. George G. Wright, Chemical Corps, Biological Laboratories, Camp Detrick, Frederick, Maryland. He has recently[3] described the preparation of this material which stimulates immunity in animals to later challenge with virulent vs. anthracis.

Dephosphorylation of ATP.—The enzyme activity was measured in a micro system patterned after DuBois and Potter.[4] As a rule, the test system was composed of 0.1 ml of the WBC (n.f.) homogenate, 0.15 ml of 0.013 *M* of the substrate solution, 0.05 ml of 0.04 *M* $MgCl_2$, 0.15 ml of the buffer (2-amino-2-hydroxymethyl-1, 3-propanediol) solution. The total volume of the assay system was 0.65 ml in a 10×55 mm test tube. The mixture was incubated for 10 minutes at 37.5± 0.5 C. At the end of the incubation period, the reaction was stopped by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid. The tubes were immediately centrifuged in a cold room. Three-tenths ml of the supernatant was used for orthophosphate assay, by the method of Fiske and Subbarow.[5] The optical density was determined with the Beckman DU spectrophotometer, using Lowry-Bessey micro-cuvette,[6] at λ820 mμ. Tissue, reagent, and substrate blanks were run with all experiments, and the results subtracted from the experimental values.

RESULTS

I. Characterization of the enzyme ATPase

pH.—It was found from pH dependence data that there was an optimum range for the guinea pig and the rat WBC (n.f.) homogenate ATPase activity varying from pH 9.5 to 9.7 when tested in 1,3-diaminobutane[7] and 2-amino-2-methyl-1,3-propanediol[8] buffers.

Temperature.—Four investigations of ATPase activity in the guinea pig WBC (n.f.) homogenate were carried out at various temperatures. Three series of determinations were made with the rat WBC (n.f.) homogenate to define ATPase activity at various temperatures. The temperature optimum for the enzyme activity was found to be 38° C for preparations from both species.

2. Heidelberger, M., McLeod, C. M., Markowitz, H. and Roe, A. S. 1950, Improved methods for the preparation of the specific polysaccharides of pneumococcus. J. Exper. Med. **91**: 341–349.

3. Wright, G. G., Hedberg, M. A. and Feinberg, R. J. 1951, Studies on immunity in anthrax II in vitro elaboration of protective antigen by non-proteolytic mutants of *Bacillus anthracis*. J. Exper. Med. **93**: 523–527.

4. DuBois, K. and Potter, V. R. 1943, The assay of animal tissues for respiratory enzymes. III. Adenosinetriphosphatase. J. Biol. Chem. **150**: 185–195.

5. Fiske, C. H. and Subbarow, Y. 1925, The colorimetric determination of phosphorus. J. Biol. Chem. **66**: 375–400.

6. Lowry, O. H. and Bessey, O. A. 1946, The adaptation of the Beckman spectrophotometer for measurements on minute quantities of biological materials. J. Biol. Chem. **163**: 633–639.

7. Gomori, G. 1952, The complex nature of alkaline phosphatase. Biochemica et Biophysica Acta. **8**: 162–172.

8. Gomori, G. 1946, Buffers in the range of pH 6.5 to 9.6. Proc. Soc. Exper. Biol. & Med. **62**: 33–34.

Activation by ions.—The effect of calcium and magnesium ions on ATPase has been much discussed in the literature.[9] The leucocyte homogenate ATPase activity requires Mg^{++} rather than Ca^{++}. In myosin-ATPase, Ca^{++} is the activator and Mg^{++} is the inhibitor.[10] The optimum concentration of Mg^{++} for both guinea pig and rat WBC (n.f.) homogenate was 0.003 *M*. On the other hand, Ca^{++} appears to be a dispensable factor in the activity of the enzyme.

Ethylene diamine tetraacetate (EDTA).—Nearly complete inhibition of the enzyme activity takes place in the concentration of 10^{-2} *M* of EDTA.[11] The 10^{-2} *M* EDTA treated guinea pig WBC (n.f.) homogenate ATPase activity cannot be restored completely by the addition of 10^{-1} *M* Ca^{++} or Mg^{++}. But in 10^{-2} *M* EDTA treated rat WBC (n.f.) homogenate, Mg^{++} will restore its activity at about 3×10^{-3} *M*, and at 10^{-2} *M* the enzyme activity is activated 200%. Calcium ion on the other hand does not remove the 10^{-2} *M* EDTA inhibition.

Sulfhydryl groups.—In recent years, increasing attention has been paid to the role of sulfhydryl groups on enzymes. They appear to be essential for the action of considerable number of enzymes and probably participate in the regulation of enzymatic processes.[12] All enzymes can be divided into two classes: those requiring the presence of sulfhydryl groups for their actions and those not requiring this group. Whether an enzyme belongs to one or the other group may be determined by its behavior toward reagents that react with sulfhydryl or disulfide groups. One of these reagents is *p*-chloromercuribenzoate.[13] At 10^{-3} *M* concentration of the reagent, the enzyme activity was inhibited to the extent of 63% in guinea pig WBC (n.f.) homogenate and at the same concentration the ATPase activity in the rat WBC (n.f.) homogenate was inhibited by about 30%.

KCN.—Muscle ATPase has been reported to be completely insensitive to potassium cyanide in concentration up to 0.1 *M*. On the other hand Binkley, Ward and Hoagland[14] found that this concentration of potassium cyanide exerted certain inhibitory effects on ATPase activity, while a 0.01 *M* concentration increased the sulfhydryl groups in the system. They presented evidence in favor of the hypothesis that sulfhydryl groups of the myosin molecule are functional units in the cleavage of phosphate from adenosine triphosphate.

KCN in the concentration range of 10^{-6} to 10^{-3} *M* activated ATPase in WBC (n.f.) homogenates 32 to 100% for the guinea pig and about 20 to 30% for the rat.

Ascorbic acid.—According to the work of Engelhardt[10] when solutions of myosin were treated with a mild oxidizing agent, ATPase activity was diminished.

9. Novikoff, A. B., Hecht, L., Podber, E. and Ryan, J. 1952, Phosphatases of rat liver: I. The dephosphorylation of adenosinetriphosphate. J. Biol. Chem. **194**: 153–170.
10. Engelhardt, B. A. 1946, Adenosinetriphosphatase properties of myosin, Advances in Enzymology, vol. 6, Interscience Publishers, Inc., New York, pp. 147–191.
11. Hunters, S. H., Provasoli, L., Schatz, A. and Haskins, C. P. 1950, Some approaches to the study of the role of metals in the metabolism of microorganism. Proc. Am. Phil. Soc. **94**: 152–170.
12. Barron, E. S. G. 1951, Thiol groups of biological importance, Advances in Enzymology, vol. 11. Interscience Publishers, Inc., New York, pp. 201–266.
13. Barron, E. S. G. and Singer, T. P. 1945, Studies on biological oxidation: XX. Sulfhydryl enzymes in fat and protein metabolism. J. Biol. Chem. **157**: 241–253.
14. Binkley, F., Ward, S. M. and Hoagland, C. L. 1944, Reversible inactivation of the adenosinetriphosphatase activity of myosin preparation with copper and cyanide. J. Biol. Chem. **155**: 681–682.

When the oxidized myosin was treated with reducing agents such as glutathione, and to a lesser extent, ascorbic acid, both of which are known to reduce oxidized sulfhydryl groups, a considerable increase in ATPase activity resulted. Nungester and Ames[1] demonstrated a relationship between the ascorbic acid content of guinea pig polymorphonuclear leucocytes and phagocytic activity. They reported that leucocytes with low ascorbic acid content were lacking in phagocytic activity.

In this investigation ascorbic acid activated ATPase from rat and guinea pig WBC (n.f.) homogenates by 1400 and 400%, respectively.

Glutathione.—Ascorbic acid and glutathione are biochemically related. Glutathione prevents inactivation of the sulfhydryl groups of enzymes. When oxidized, the ATPase activity of myosin solution is diminished; however, the activity is restored with reducing agents such ascorbic acid, glutathione, etc.[10] Thus, the inactivation and reactivation of myosin-ATPase results from the disappearance and restitution of sulfhydryl groups.

Glutathione was found to activate the ATPase in guinea pig WBC (n.f.) homogenate about four times as much as in the rat homogenate at concentrations of 10^{-3} to 10^{-2} *M*.

II. A comparative study of the effect of pneumococcal polysaccharides from virulent and avirulent type I D. pneumoniae *and also* B. anthracis *antigen on the ATPase activity of WBC (n.f.) homogenates of the guinea pig and the rat*

The lethal dose of type I pneumococci is a million times greater for the guinea pig than the rat. This difference stimulated comparative investigation of the action of a specific polysaccharide, SI, on the ATPase activity of WBC (n.f.) homogenate from the susceptible rat, and the resistant guinea pig. For similar reasons the effect of a specific polysaccharide, SI-L4, from an avirulent strain of type I pneumococci was investigated. With respect to *B. anthracis* the guinea pig is the susceptible host and the rat is resistant. Assuming that the protein antigen of Wright[3] was related to virulence of *B. anthracis*, since it stimulated immunity to challenge with virulent organisms, a study of the effect of this antigen on ATPase activities in the WBC (n.f.) homogenate from the rat and guinea pig was made.

SI and SI-L4 on ATPase activity.—The effect of SI on ATPase in guinea pig WBC (n.f.) homogenate was to double the activity of the enzyme. On the other hand, the ATPase activity of rat WBC (n.f.) homogenate was decreased by about 47% (table 1).

Polysaccharide from an avirulent type I *D. pneumoniae* was designated SI-L4. The avirulent strain of penumococcus type I was obtained from a rat lung which had been inoculated with a virulent strain of type I pneumococcus (strain 76) by Alonso.[15] When he tested it for virulence, a dose of 10^9 organisms failed to kill the rat. Passing the same quantity of organisms through the peritoneal cavity of rats did not increase its virulence.

The effect of the polysaccharide from the avirulent pneumococcus type I on ATPase activity of the rat and the guinea pig WBC (n.f.) homogenate was studied. The result was the reverse of the inhibitory effect of SI on ATPase

15. Alonso, D. 1952, A comparative study of the metabolism of leucocytes from guinea pigs and rats. Doctoral Dissertation, Dept. of Bacteriology, Univ. of Mich

TABLE 1.—*The effect of SI on ATPase activity of the guinea pig and the rat leucocyte (n.f.) homogenates.*

Concentration during 120 minutes preliminary incubation at 25 C	Concentration during 10 minutes incubation at 37.5 C	Percent change from control	
		Guinea pig	Rat
6.5×10^{-1}%	1×10^{-1}%	29.0*	−47.0*
6.5×10^{-2}%	1×10^{-2}%	82.5	−43.0
6.5×10^{-3}%	1×10^{-3}%	106.0	−47.5
6.5×10^{-4}%	1×10^{-4}%	82.5	−47.5
6.5×10^{-5}%	1×10^{-5}%	106.0	−30.0
6.5×10^{-6}%	1×10^{-6}%	106.0	−24.0
6.5×10^{-7}%	1×10^{-7}%	94.0	−26.5

* The figures represent the mean values of 8 determinations.

activity in the rat WBC (n.f.) homogenate. The enzyme was activated from about 90 to 150%. SI-L4 did not affect the ATPase activity of guinea pig WBC (n.f.) homogenate.

Anthrax antigen on ATPase activity.—Metchnikoff[16] found that in animals naturally resistant to anthrax, such as the rat, phagocytosis occurred promptly and all the ingested bacilli were engulfed by leucocytes, while almost no phagocytosis occurred in susceptible species, such as guinea pig.

A study was made of the effect of *B. anthracis* antigen on the ATPase activities of WBC (n.f.) homogenate of the resistant and susceptible animals, respectively.

TABLE 2.—*The effect of* B. anthracis *antigen on ATPase activity of the guinea pig and the rat leucocyte (n.f.) homogenates.*

Concentration during 120 minutes preliminary incubation at 25 C	Concentration during 10 minutes incubation at 37.5 C	Percent change from control	
		Guinea pig	Rat
6.5×10^{-2}%	1×10^{-2}%	None*	330*
6.5×10^{-3}%	1×10^{-3}%	−27	130
6.5×10^{-4}%	1×10^{-4}%	−33	11
6.5×10^{-5}%	1×10^{-5}%	−25	6
6.5×10^{-6}%	1×10^{-6}%	−21	8

* The figures represent the mean values of 6 determinations.

16. Metchnikoff, E. 1892, Leçons sur la Pathologie Comparee de l' Inflammation. Paris, Masson et Cie, 239 pp.

TABLE 3.—*The effect of bacterial fractions (SI, SIII, SI-L4, and anthrax antigen) on the ATPase activity of guinea pig, rat, and rabbit leucocyte nuclei-free homogenate.*

Leucocyte (n.f.) homogenate of:	SI	Anthrax antigen	SIII	SI-L4*
Guinea pig:				
a. Susceptible to: *B. anthracis*				
b. Resistant to *D. pneumoniae* type I and III	+	−	+	±
Rat:				
a. Susceptible to *D. pneumoniae* type I and III	−	+	−	+
b. Resistant to *B. anthracis*				
Rabbit:				
a. Susceptible to *D. pneumoniae* type I and *B. anthracis*	−	−	+	+
b. Resistant to *D. pneumoniae* type III				

* SI-L4, polysaccharide prepared from avirulent strain of *D. pneumoniae;* a dose of 10^8 organisms injected intraperitoneally failed to kill any of the hosts.
+, enzyme activity enhanced.
−, enzyme activity inhibited.

Table 2 shows that *B. anthracis* antigen activated the ATPase activity in the rat WBC (n.f.) homogenate. The converse was true for the enzyme from the guinea pig. The activity was inhibited by as much as 33%.

III. The effect of bacterial fractions on the ATPase activity of rabbit WBC (n.f.) homogenate

This additional study was made because rabbit is a host susceptible to type I *D. pneumoniae* but naturally resistant to type III.[17]

SI inhibited the enzyme preparation by approximately 40% when the concentration of the polysaccharide was about 0.1%. SIII and SI-L4 enhanced the enzymatic activity of the homogenate. The results are summarized in table 3.

17. Tillet, W. S. 1927, Studies on immunity to pneumococcus mucosus (type III). II. The infectivity of Type III pneumococcus for rabbits. J. Exper. Med. **45**: 1093–1106.

TABLE 4.—*The effect of bacterial fractions (SI, SIII, SI-L4 and anthrax antigen) on the ATPase activity of guinea pig, rat, rabbit and mouse spleen homogenate.*

Spleen homogenate of:	SI	Anthrax antigen	SIII	SI-L4*
Guinea pig:				
a. Susceptible to: *B. anthracis*				
b. Resistant to: *D. pneumoniae* type I and III	+	−	+	+
Rat:				
a. Susceptible to: *D. pneumoniae*	−	+	−	+
b. Resistant to: *B. anthracis*				
Rabbit				
a. Susceptible to: *D. pneumoniae* type I and *B. anthracis*	−	−	+	+
b. Resistant to: *D. pneumoniae* type III				
Mouse:				
a. Susceptible to: *D. pneumoniae* type I and III and also *B. anthracis*	−	−	−	+

* SI-L4, polysaccharide prepared from avirulent strain of *D. pneumoniae*; a dose of 10^9 organisms injected intraperitoneally failed to kill any of the hosts.
+, enzyme activity enhanced.
−, enzyme activity inhibited.

IV. The effect of SI, SIII, and anthrax antigen on the ATPase activity of the spleen homogenate of the guinea pig, rat, rabbit, and mouse

The question arose whether or not other tissues of the reticuloendothelial system might be similarly affected by bacterial antigen as that of the leucocyte homogenate. Substantial data were obtained showing that the spleen ATPase activity of the given host is similarly enhanced or inhibited by bacterial fractions as the leucocyte ATP hydrolyzing enzyme. The results are tabulated in table 4.

DISCUSSION

It is generally accepted that the activity of any cell depends on the types and relative activities of its enzymes. The answer to whether a given fraction of a virulent parasite influences enzyme systems (particularly the energy releasing ones) of a resistant host differently than those of a susceptible animal is an important factor in understanding host resistance.

In the present investigation certain factors and conditions which have been known to effect phagocytosis also affect ATPase activity in the WBC (n.f.) homogenates. Furthermore, substantial similarities were found between the leucocyte homogenate and the spleen homogenate ATPase. For instance, the homogenates were activated by Mg^{++}. Magnesium ion is claimed to "increase both the ameboid activity and the number of ingested particles per cell"[18] Kielley and Kielley[19] reported that mouse liver mitochondrial ATPase was magnesium-activated. Novikoff, Hecht, Podber, and Ryan[9] reported a magnesium activated ATPase in the isolated mitochondria of rat liver.

Nungester[20] reported that the addition of cyanide had no effect on leucocyte phagocytic activities and stated: ". . . , phagocytes, at least guinea pig exudate cells, can phagocytize in an atmosphere of nitrogen or in the presence of cyanide in concentrations sufficient to block aerobic metabolism, according to results from our laboratory. . . . It is teleologically significant that these scavenger cells can operate in low oxygen tension." In the present investigation KCN in concentrations of 10^{-6} to 10^{-3} *M* activated ATPase in

18. Kleine, E. 1952, Minutes of formed elements meeting, Univ. Lab. of Phys. Chem. Related to Med. and Pub. Health, Harvard Univ., Boston, May 15, 1952; quoted by Tullis, J. L. 1953, Preservation of Leukocytes. Blood, **8**: 564.
19. Kielley, W. W. and Kielley, R. K. 1953, A specific adenosinetriphosphatase of liver mitochondria. J. Biol. Chem. **200**: 213–222.
20. Nungester, W. J. 1951, Resistance to infectious diseases. Bact. Rev. **15**: 105–129.

leucocyte (n.f.) homogenates. Binkley, Ward, and Hoagland[14] found a similar situation with respect to myosin-ATPase activity. Leucocytes seem to have great propensity for deriving their energy from an anaerobic glycolytic cycle. Probably for these reasons leucocytes can continue their phagocytic activities in low oxygen tension as well as in the presence of cyanide.

Fractions of pathogens believed to be associated with virulence of certain parasites as pneumococcus polysaccharide and anthrax antigen have been shown in this study to alter ATPase activity of leucocytes and spleen even in low concentrations. Thus, it is tempting to postulate that the effect exerted by such fractions on ATPase activity of phagocytic cells of a given host may be an important factor determining whether or not the host will be susceptible to the parasite.

SUMMARY

1. Adenosinetriphosphatase activity has been found in the leucocyte, nuclei-free, homogenate of the guinea pig, rat, and rabbit; and in spleen homogenate of guinea pig, rat, rabbit, and mouse.

2. The enzyme has been partially characterized.

3. SI activated the ATPase from guinea pig (resistant host) leucocytes 100% or more above the control in the concentration range of 10^{-6} to 10^{-3}%. The ATPase from rat (susceptible host) leucocytes was inhibited by 47% by concentrations of 10^{-6} to 10^{-1}% of SI. The rabbit leucocyte ATPase was inhibited by SI but activated by SIII.

4. SIII also enhanced the enzyme activity of guinea pig (resistant) leucocyte ATPase. This pneumococcal polysaccharide inhibited the enzyme activity of rat and mouse (susceptible hosts) tissue homogenates.

5. *B. anthracis* antigen inhibited the enzyme activity in homogenates of cells of susceptible hosts (guinea pig, rabbit, and mouse), but activated ATPase activity of homogenates prepared from the rat, which is naturally resistant to anthrax.

COMPARATIVE STUDY OF HOST RESISTANCE OF GUINEA PIG AND RAT

III. PNEUMOCOCCIDAL EXTRACTS FROM THE LIVER AND SPLEEN OF THE SUSCEPTIBLE RAT AND THE RESISTANT GUINEA PIG

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There have been numerous reports of antibacterial materials extracted from various organs from a number of different animal species.[1–7] In some cases there has been a definite attempt to correlate the existence of the antibacterial agent in the tissues with the resistance of the animal to the disease.[8–10] On the whole, however, from the existing evidence such a correlation has been difficult.

This present investigation is concerned with the isolation, partial purification and characterization of a pneumococcidal agent from the liver and spleen of the resistant guinea pig and the susceptible rat.

METHODS AND MATERIALS

Animals.—The guinea pigs and white rats (Sprague-Dawley strain) used in these experiments were normal adult individuals which had not been previously injected with pneumococcus antigen or antibody. All animals were fasted 18 to 24 hours previous to sacrificing.

Organism.—The test organism was *Diplococcus pneumoniae* type I strain 76. Virulence was maintained by animal passage through rats at intervals of about 3 weeks. The virulence of the organism was such that a 1×10^{-7} dilution of a 6 hour culture (approximately 10 to 100 organisms) was lethal for rats by intraperitoneal inoculation. The LD_{50} for guinea pigs by the same route of inoculation was between 1×10^{7} and 1×10^{8} organisms.

For routine subculture a 0.1 ml inoculum was introduced into 10 ml of Difco brain heart infusion browth (BHI). This was incubated for 6 hours at 37 C, and then stored at 2 to 5 C. A culture was never used after 24 to 48 hours storage.

Harvesting of tissues.—Animals were sacrificed by means of ether. The tissues were removed

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1. Pirie, A. 1935, The effect of extracts of pancreas on different viruses. Brit. J. Exper. Path. **16**: 497–502.
2. Nutini, L. G. and Kreke, C. W. 1942, Toxic effect of splenic extracts on *Streptococcus hemolyticus*. J. Bact. **44**: 661–666.
3. Nutini, L. G. and Lynch, Sr., E. M. 1945, Effect of tissue extracts in controlling *Staphylococcus aureus* infections. Nature, **156**: 419–420.
4. Nutini, L. G., Kreke, C. W. and Schroeder, Sr., M. P. 1945, Further studies on the effects of spleen extracts on bacteria. J. Bact. **50**: 177–185.
5. Nutini, L. G. and Lynch, Sr., E. M. 1946, Further studies on effects of tissue extracts on *Staphylococcus aureus*. J. Exper. Med. **84**: 247–261.
6. Bloom, W. L., Watson, D. W., Cromartie, W. J. and Freed, M. 1947, Studies on infection with *B. anthracis:* IV. Preparation and characterization of an anthracidal substance from various animal tissues. J. Infect. Dis. **80**: 41–52.
7. Bloom, W. L. and Blake, F. G. 1948, Studies on an antibacterial polypeptide extracted from normal tissue. J. Infect. Dis. **83**: 116–123.
8. Rothman, S., Smiljanic, A., Shapiro, A. L. and Weitkamp, A. W. 1947, The spontaneous cure of tinea capitis in puberty. J. Invest. Dermat. **8**: 81–98.
9. Kligman, A. M. and Ginsberg, D. 1950, Immunity of the adult scalp to infection with *Microsporum audouini*. J. Invest. Dermat. **14**: 345–358.
10. Bloom, W. L., Hudgins, P. C. and Cummings, M. M. 1953, Tuberculostatic substances extracted from normal rat spleen. J. Infect. Dis. **92**: 70–73.

aseptically and placed immediately in a mixture of ethanol and dry ice. The gall bladder, in the case of the guinea pig, was removed intact and discarded before freezing the tissue. Since the white rat does not possess a gall bladder no such precaution was necessary for this species.

Measuring antibacterial activity of tissue extracts.—The tube dilution method was used for assaying antibacterial activity. After incubating the inoculum with the extract suspended in BHI broth for 6 hours at 37 C, plate counts were made. The minimum concentration of extract causing sterilization of a maximum inoculum in 6 hours was taken as the end point for bactericidal activity.

Preparation of tissue extract.—Preliminary experiments, using the filter pad technique, indicated an antipneumococcal property in minced livers, liver homogenates and especially in concentrated aqueous extracts of liver. This latter material, however, was unsuitable for quantitative assay. A 50% ethanol soluble extract was found to be more satisfactory. It was prepared as follows. One hundred grams of frozen liver were homogenized in 100 ml of sterile distilled water at room temperature for 2 minutes in a waring Blender, then held at 5 C for 1 hour. Ethanol was added to give a final concentration of 50% by volume and the mixture allowed to stand 2 hours at room temperature. A heavy precipitate formed which was filtered off and discarded. The clear, greenish yellow filtrate became cloudy on standing. The alcohol was removed by boiling under reduced pressure at 40 to 50 C. This latter step required from 4 to 18 hours depending upon the original volume of the material. During this process the material developed a marked turbidity. After centrifugation at approximately 4000 G for 1 hour, the supernatant, which was still distinctly turbid, was shell frozen and dried under vacuum. The dried residues were stored in sterile bottles in a desiccator. Aseptic precautions were followed throughout the procedure.

EXPERIMENTAL

Preliminary investigation with crude aqueous extracts of liver, as previously mentioned, indicated a marked difference in the antibacterial property of the material obtained from the resistant guinea pig and the susceptible rat. It was possible that the source of the observed antipneumococcal activity of the tissue extracts might be the blood in the highly vascular organs (liver and spleen), hepatic bile, or a product derived from the tissues during processing or by bacterial enzyme action during incubation of extract with the inoculum. However, ethanol extracts of blood failed to show any antipneumococcal activity. The active agent in the extract was shown to have properties different from bile (extract non-Seitz filterable, inactivated by serum and quantitatively more active than bile), and finding the spleen to yield active extracts further differentiated the active factor from bile.

That the antipneumococcic agent in the extracts was not an enzymatically derived product was shown by the fact that incubation of homogenates at 37 C before extraction did not enhance the activity of the final product. Furthermore, boiling for 30 minutes to drive off the ethanol in the solution, instead of the longer procedure of distilling it off under reduced pressure, failed to produce any change in the titer of the final product.

A study of the time and mode of action of the antipneumococcal extract indicated that the pneumococci were killed almost instantly, especially in the suspensions of higher concentrations of extract. The colony counts were markedly reduced in 5 minutes (from 4×10^7 to 1×10^1), although intact cells were still observed. However, after 2 hours the cells appeared to be completely lysed. The immediate killing action of the extract is not compatible with the hypothesis that a toxic factor is released from the extract through enzymatic action of bacterial enzymes.

Pneumococcidal activity of tissue extracts.—Fifty percent ethanol soluble extracts of pooled liver of the guinea pig and of the rat were prepared and tested for pneumococcidal activity. The number of animals represented in the pools varied and the extracts were not

TABLE 1.—*A comparison of the pneumococcidal activity of 50% ethanol-soluble extracts prepared from pooled guinea pig liver and from pooled rat liver.*

Number of animals represented		Guinea pig		Rat	
Guinea pig	Rat	Minimum concentration destroying maximum inoculum, mg per ml	Maximum inoculum completely destroyed in 6 hours	Minimum concentration destroying maximum inoculum, mg per ml	Maximum inoculum completely destroyed in 6 hours
10	8	1.75	10^7	50	10^5
14	8	0.1	10^7	50	10^5
14	14	1.0	10^7	10	10^4
14	14	0.1	10^7	50	10^5
14	14	0.3	10^7	50	10^5
14	14	0.3	10^7	10	10^5
6	14	7.0	10^5	10	10^5
8	6	0.01	10^5	10	10^5
16	12	3.0	10^7	100	10^7
10	14	0.1	10^7	10	10^5
20	32	1.0	10^7	10	10^5
2	8	1.0	10^7	50	10^5

all prepared or tested at the same time. However, treatment of the animals (18 to 24 hours fasting) and method of processing the material was the same in all cases.

An examination of table 1, which summarizes these data, reveals both a variation of extract activity from tissues within the species and a marked difference in activity of tissue extracts between the guinea pig and the rat. Whereas the extracts of guinea pig liver exhibited a consistently high degree of pneumococcidal activity, the activity of the rat extracts was of a very low order.

The observation that there was variation within the species in the activity of the extracts prepared from pooled tissues, prompted an investigation of the pneumococcidal effect of extracts prepared from a number of individuals of the two species.

A total of ten 50% ethanol soluble extracts were prepared from the livers of ten guinea pigs. The livers of ten rats were treated in the same manner. Generally, as many as six extracts, representing three guinea pigs and three rats, were prepared and tested at the same time under identical conditions.

Table 2 summarizes the results of these individual animal tests. In extracts prepared from ten guinea pigs six, in a relatively low concentration, completely destroyed an inoculum of 10^7 organisms. No rat extract, of the ten tested, in the same low concentration or even in much higher concentration, destroyed a similar inoculum.

It was apparent from these data that there is, within the species, a variation of the pneumococcidal activity of liver extracts. Furthermore, it was again observed that the extracts of guinea pig livers are significantly superior to those of rat livers in pneumococcidal effect. A record was kept of individual weights of the livers used in these experiments, but no correlation could be drawn between the weight of the tissue and its pneumococcidal activity.

This type of study was extended to an extract from spleens obtained from

TABLE 2.—*A summary of the pneumococcidal activity of 50% ethanol-soluble liver extracts prepared from ten individual rats and guinea pigs.*

Minimum concentration destroying maximum inoculum, mg per ml	Maximum inoculum completely destroyed	Number of individual animals having this effect	
		Guinea pigs	Rats
6.25	10^7	6	0
25.0	10^7	1	0
25.0	10^5	0	1
50.0	10^5	1	0
50.0	10^5	2	1
50.0	10^5	0	4
50.0	0	0	4

TABLE 3.—*A comparison of the pneumococcidal activity of three 50% ethanol-soluble extracts of the liver and spleen of rats and guinea pigs.*

Sample no.	Guinea pig				Rat			
	*Liver		*Spleen		Liver		Spleen	
	†1	‡2	1	2	1	2	1	2
1	0.1	7×10^7	100	7×10^7	100	7×10^8	100	7×10^8
2	0.1	3×10^7	0.1	3×10^7	50	3×10^8	50	3×10^8
3	1.0	5×10^7	1.0	5×10^7	10	5×10^8	10	5×10^8

* Pooled tissues of 14 animals.
† Minimum concentration destroying maximum inoculum, mg per ml.
‡ Maximum inoculum completely destroyed in 6 hours.

14 guinea pigs. An extract of livers from the same animals were examined at the same time. Similar extracts were prepared from the pooled spleens and livers of 14 rats. The extracts of both species were tested simultaneously. This experiment was repeated three times; thus three different lots of pooled extracts, representing a total of 42 animals in each species, were prepared and tested for pneumococcidal effect.

The data from these experiments (table 3) show in each species a close parallelism in pneumococcidal effect of extracts prepared from the liver and the spleen, but the extracts of both of these tissues in the case of the guinea pig were superior in activity to those of the rat.

Fractionation of dried 50% ethanol-soluble extract.—The 50% ethanol-soluble extract was, admittedly, a very impure fraction. To gain information concerning the nature of the active principle and to study the basis of the difference in the activity of the extracts of the two species it was necessary to attempt further purification.

Certain properties of the 50% ethanol-soluble extract, such as the fact that it was coctostabile, only weakly soluble but easily suspendable in an aqueous medium, soluble in 50% ethanol and non-Seitz filterable, suggested that the active principle might be a lipid or lipid complex.

The fractionation technique finally developed was as follows. A weighed amount of dried 50% ethanol-soluble material was further extracted with purified absolute ethanol by grinding on the ball mill for 18 to 24 hours. The insoluble material was removed by centrifugation at approximately 4000 G for 1 hour. An aliquot of this ethanol extracted fraction was dried and tested. The remainder was dried by boiling under reduced pressure in a 40 to 50 C water bath. To this was added a measured amount of purified ethyl ether. The ether extraction was carried out by allowing the mixture to stand at 5 C for 18 to 24 hours. The ether-insoluble fraction was removed by centrifugation. An aliquot of the ethanol-ether extracted fraction was also dried and tested, the remainder being concentrated by boiling under reduced pressure. This concentrated ethanol-ether extracted fraction was then added to an excess of cold purified acetone. The precipitate which formed was removed by centrifugation. The clear supernatant was dried by boiling under reduced pressure.

In testing for pneumococcidal activity the three fractions were suspended in BHI broth using a Potter-Elvejhem type glass homogenizer. In order to have a basis for comparison of the pneumococcidal activity of each fraction with that of the dried crude 50% ethanol extract, the final concentration of each suspension was made equivalent to that of the original dried material.

Such fractions were prepared from

TABLE 4.—*A comparison of the pneumococcidal activity of organic solvent fractions of the 50% ethanol-soluble extracts of guinea pig and rat liver.*

Species	Equivalent concentration, mg per ml	Initial inoculum	*50% ethanol-soluble extract	Ethanol extracted fraction	Ethanol, ether extracted fraction	Ethanol, ether, acetone extracted fraction
Guinea pig	10	3×10^7	0†	0	0	0
	1	3×10^7	0	0	0	0
	0.1	3×10^7	1×10^1	9×10^5	1×10^4	5×10^5
	0.01	3×10^7	2×10^5	6×10^5	1×10^5	5×10^5
Rat	10	3×10^7	1×10^5	5×10^5	0	0
	10	3×10^6	6×10^6	0	0	0
	10	3×10^5	0	0	0	0
	1	3×10^7		2×10^5	2×10^5	1×10^5
	0.1	3×10^7		1×10^5	1×10^5	3×10^5

* The pH of all fractions in suspension had a range of 7.1 to 7.3.
† These figures represent colonies per ml after incubation of fractions with the inoculum for 6 hours at 37 C.

dried guinea pig and rat liver extracts. These fractions, with the original 50% ethanol-soluble extract, were tested simultaneously for pneumococcidal activity. Three experiments were carried out for each species on three different lots of extract. Representative data are recorded in table 4.

These experiments revealed an interesting difference in the fractions obtained from the two species. Whereas the same degree of activity was evident in all the guinea pig fractions, purification of the 50% ethanol-soluble extract of rat liver markedly enhanced its pneumococcidal effect. There had been some indication of this in earlier tests and it was again shown consistently in these three experiments. It should be pointed out, however, that in all three experiments there was still a quantitative difference between the pneumococcidal activity of the final fraction (ethanol-ether-acetone extracted) of the guinea pig and rat livers. In all three instances there was a tenfold difference in favor of the guinea pig liver fraction.

DISCUSSION

The primary goal of the present investigation was to search for pneumococcidal substances in the tissues of a resistant and susceptible host. The data which have been presented in this study definitely point to the presence of a pneumococcidal factor in certain tissues of the resistant species. In the susceptible species the factor is either present in low concentration, is present but inhibited or is a different entity of inferior pneumococcidal power. As to which of these situations is the most likely will be discussed.

All of the evidence, to date, has been obtained from in vitro studies and hence no evidence can be presented to indicate a possible in vivo function of this material. However, it seems reasonable to believe that this pneumococcidal factor does function in vivo as a factor in host resistance.

The fact that this active agent can be readily extracted with water or with 50% ethanol without previous incubation of the tissues and that it is not dependent on blood or bile for its activity is suggestive that this material exists as such in the tissues of the host. This is the logic followed by Ponder,[11] who states that certain lytic substances, which have been extracted from blood and tissues not previously incubated, are present and function as such in normal blood and plasma and "by inference in normal tissue." If this does follow then it can be postulated that this agent

11. Ponder, E. 1952, Separation and assay of lysins and lysin-inhibitor complexes in blood and tissues, J. Gen. Physiol. 35: 361–374.

may be present in the phagocytic cells of the liver of the guinea pig and is able to destroy the phagocytized pneumococci which have escaped the other defenses of the body. Whereas in the cells of the rat this agent is either present in very low concentration, or is present with an inhibitor. Thus the rat liver would be less able to dispose of pneumococci which had evaded the other defenses.

A comparison of the pneumococcidal strength of the fractions obtained by the stepwise breakdown of the 50% ethanol-soluble extract seems to give some insight into the basis for the difference in the activity of extracts prepared from the tissues of the two species. The ethanol-ether-acetone extracted fraction obtained from the rat liver in equivalent concentration, was far more active than the original dried material. This was not found to be true in the case of the guinea pig as all fractions, in equivalent concentration, were equally as active as the original extract. Apparently then, in the 50% ethanol-soluble rat liver extract (and by inference in the liver itself) there exists an inhibitor complex which interferes with the pneumococcidal activity of the active agent. The over-all activity of the extract which has been shown to vary to some extent among individuals of the species, would then depend upon the ratio of the active agent to the inhibitor or inhibitor complex.

An analogy for such a hypothesis may be found in the work of Tyler[12–14] and Ponder.[11,15] In connection with hemolytic systems they both postulate the in vivo existence of an easily dissociable lysin-inhibitor complex and further that the hemolytic activity is dependent on the concentration of the free lysin together with the "buffering" effect of the inhibitor.

Although there is no evidence thus far that such an inhibitory mechanism operates in the guinea pig liver, fractionation of guinea pig extracts of relatively low pneumococcidal strength might reveal the existence of a similar mechanism.

The pneumococcidal agent has not been sufficiently purified to permit more than a presumptive chemical characterization. However, on the basis of solubilities on 50% ethanol and in purified organic solvents, and also on the basis of coctostability, and certain apparent physical characteristics, such as inactivation by serum, adsorption onto protein, and the oily consistency of the acetone soluble fraction, the active factor appears to be most closely related to the lipids.

SUMMARY

A tissue extract, bactericidal for the type I pneumococcus, was prepared from the liver and the spleen of the guinea pig and rat.

The pneumococcidal agent was shown to be weakly soluble in water, considerably more soluble in 50% ethanol, resistant to boiling, non-Seitz filterable, and inactivated by serum.

The activity of this material was found to be independent of any pneu-

12. Tyler, D. B. 1949, An autohemolytic agent in fetal liver extracts, Proc. Soc. Exper. Biol. & Med. **72**: 491–495.
13. Tyler, D. B. 1950, The hemolytic and antihemolytic activities of various centrifugally separated fractions of adult and fetal liver cells. Science, **112**: 456–459.
14. Tyler, D. B. 1951, Naturally occurring inhibitors and lysins in various centrifugally prepared extracts of adult and fetal guinea pig tissue extracts. Am. J. Physiol. **164**: 467–475.
15. Ponder, E. 1951, Tissue hemolysins as lysin-inhibitor complexes, J. Gen. Physiol. **34**: 551–565.

mococcidal activity of the blood or bile. Also, it was shown that the active agent is not a product derived from the tissues by enzymatic action.

Fifty percent ethanol soluble extracts prepared from the pooled livers of guinea pigs were found to be significantly more pneumococcidal than similar extracts prepared from pooled rat livers.

Extracts prepared from the tissues of individuals within each species were variable in pneumococcidal activity.

Spleenic extracts from guinea pigs have greater pneumococcidal activity than those of rats.

The 50% ethanol-soluble liver extract was partially purified by stepwise fractionation with organic solvents. The active agent was found in the ethanol-ether-acetone extracted fraction.

Indirect evidence was presented for the existence of an inhibitor complex in the 50% ethanol-soluble extract of rat liver. No such inhibitory mechanism was demonstrated in similar extracts of guinea pig liver.

THE INFLUENCE OF AGE ON THE INTENSITY OF INFECTION WITH *PLASMODIUM BERGHEI* IN THE RAT

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Shortly after the inception of studies on *Plasmodium berghei* in this laboratory, it became apparent that susceptibility of rats to infection was dependent on age, both parasitemia and mortality decreasing strikingly as the age of the host increased. This phenomenon had been noted earlier in rats by Galliard and Lapierre,[1] by Hsu and Geiman,[2] and more recently by Zuckerman and Yoeli.[3] A similar age-susceptibility relationship had also been observed in inbred mice by Greenberg et al,[4] and in mice and hamsters by Hsu and Geiman.[2] Since the data presented in these works were somewhat incomplete and not wholly applicable to either the strain of parasite or rat in use in this laboratory, it seemed desirable to explore age-susceptibility relationships under the conditions prevailing locally. At the completion of this investigation, attention was directed toward an explanation for the differences in susceptibility of rats of various ages. Earlier work by one of us,[5–7] demonstrating that the intensity of parasitemia in mice was directly related to the functional state of the erythropoietic tissue and in particular to the availability of reticulocytes, focused attention on the possibility that the age-susceptibility relationships as observed in the rat had a similar basis. A study was designed to test this possibility. The present report deals with the results of this study and the earlier investigation of the general age-susceptibility phenomenon.

GENERAL METHODS

The Kasapa strain of *P. berghei* was used in this study. This parasite was obtained on November 11, 1953, from Dr. G. Robert Coatney, National Institutes of Health, who had in turn obtained it from Dr. I. H. Vincke in February of 1952 and carried it thereafter by blood-passage in white mice. Since acquisition by our laboratory, the parasite has been passed biweekly in 60 to 80 g rats by the intraperitoneal inoculation of whole blood. For both passage and experimental purposes, only *Bartonella*-free Sprague-Dawley female rats have been used. These animals have been maintained in individual cages in air-conditioned quarters at 78 to 80 F and have been fed a diet of finely ground Purina Dog Checkers and water ad libitum.

For experimental purposes animals were infected intravenously with about 500,000 parasites. Blood from donor rats was drawn by cardiac puncture early in the infection (not past the 6th day), properly diluted with saline, and injected into a tail vein of the recipient. Preparations for red cell counts, reticulocyte counts, and parasite counts were made daily from tail blood, starting one day after inoculation and continuing until parasites could no longer be found on thick films. Parasites, parasitized erythrocytes, parasitized reticulocytes, and reticulocytes were determined as either the number per cmm of blood or the number per 10,000 rbc.

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1. Galliard, H. and Lapierre, J. 1951, Bull. Soc. path. exot. **44**: 185–194.
2. Hsu, D. Y. M. and Geiman, Q. M. 1952, Am. J. Trop. Med. **1**: 747–760.
3. Zuckerman, A. and Yoeli, M. 1954, J. Infect. Dis. **94**: 225–236.
4. Greenberg, J., Nadel, E. M. and Coatney, G. R. 1953, J. Infect. Dis. **93**: 96–100.
5. Singer, I. 1953, J. Infect. Dis. **92**: 97–104.
6. Singer, I. 1954, J. Infect. Dis. **94**: 159–163.
7. Singer, I. 1954, J. Infect. Dis. **94**: 164–172.

In estimating reticulocytes a drop of blood was added to a drop of 1% brilliant cresyl blue solution containing 0.85% sodium chloride and 0.4% sodium citrate in the concavity of a culture slide. This was stirred with a glass rod and permitted to stand in a wet chamber for 10 to 20 minutes. Afterwards the mixture was stirred again and a drop, measured from a capillary pipette, placed on a glass slide. A smear was made, dried, fixed in methanol, and stained with Giemsa in the usual manner.

EXPERIMENTAL

1. General age-susceptibility relationships

Table 1, which summarizes results obtained over the past 7 months, shows that parasitemia and survival were inversely related to the age of the rat. Rats younger than 28 days or less than 60 g in weight had a mortality rate in excess of 90%, while animals over 49 days old or 134 g were able to control the infection completely. Survival, in turn, depended on parasitemia. Almost all animals with fatal infections exhibited parasite counts over 10,000 per 10,000 erythrocytes, while in survivors parasitemias rarely rose above this figure. Among survivors age did not seem to affect either the time when peak parasitemia occurred, or the length of the primary attack. However, animals which died showed a delayed peak parasitemia followed by a plateau in numbers of parasites.

2. The relation of susceptibility to age and to hematopoietic activity

The relationship of age and susceptibility to infection, as described above, to the capacity of the rat to form reticulocytes was examined in a detailed study on 59 animals of varying ages. Table 2 shows the distribution and disposition of these animals and compares growth in control and infected rats. The two youngest groups showed increases

TABLE 1.—*P. berghei infections in rats of varying age.*

Weight range, grams	Weight at infection, grams	Estimated age, days	Number of rats	Data on survivors					Data on fatalities				
				Number of fatalities	Percent of fatalities	Peak parasitemia		Duration of primary attack, days	Peak parasitemia		Death		
						Number of parasites per 10,000 rbc	Day after patency		Number of parasites per 10,000 rbc	Day after patency	Number of parasites per 10,000 rbc	Day after patency	
40– 59	47.9±1.3*	28	12	11	92	8040±1327*	9.3±0.5*	21.7±1.0*	18,025±2233*	11.5±1.0*	16,554±2465*	13.6±1.0*	
60– 79	69.0±1.1	33	33	27	82	6093±443	11.0±0.4	22.7±0.6	17,783±824	14.3±0.3	15,570±866	16.3±0.3	
80– 99	91.7±0.8	39	54	31	57	4974±675	9.2±0.3	21.4±0.7	15,543±627	15.8±0.3	14,644±716	17.4±0.3	
100–119	104.3±0.8	42	21	7	33	2025±347	9.9±0.8	21.7±0.7	13,144±1600	14.3±0.4	12,003±1643	15.9±0.3	
120–139	134.5±0.7	49	15	0	0	953±549	8.9±0.8	21.5±0.4					
140–169†	159.5±0.8	57	18	0	0	899±167	10.3±0.9	22.8±0.7					
170–199‡	183.0±1.75	76	26	0	0	736±103	8.2±0.4						
200–	217.6±2.7	93+	18	0	0								

* Mean ± standard error.
† Parasitological data represent 11 animals.
‡ Parasitological data represent 10 animals.

TABLE 2.—*Disposition and mean weights of experimental animals.*

Group	Age, days	Number	Number died	Category	Average weight—grams Days after inoculation										
					0	4	7	9	11	14	16	18	21	23	25
I	30	10	9	Fatalities	61	74	86	91	85	72	71				
		5		Controls	60	74	90	99	110	122	130	138	145	149	152
II	41	6	6	Fatalities	101	115	125	131	131	110	99				
		4		Survivors	104	118	124	134	138	136	130	136	150	161	168
		5		Controls	97	115	130	138	149	155	159	166	170	178	180
III	51	9	0	Survivors	139	147	154	162	165	173	175	181	185	196	193
		5		Controls	135	145	155	171	173	179	182	186	189	197	197
IV	81	10	0	Survivors	206	211	210	210	213	220	224	226	227	235	233
		5		Controls	197	203	201	202	207	210	211	217	217	224	225

in weight which were considerably less than those seen in control animals during the first 8 days. At this time infected animals started to lose weight. This trend persisted to death among the fatal cases, while in survivors growth was resumed on the 15th day of infection. This resumption corresponded to the immediate pre-crisis period (fig. 3). Infection did not affect growth in the 51- and 81-day age groups.

The courses of infection in rats belonging to the four age groups are shown in figures 1 to 5. Parasitemia, parasitized red blood cells, parasitized reticulocytes, reticulocytes, and total red blood cells are described in terms of number per cmm of blood, while pluriparasitism is shown as the percentage of parasitized erythrocytes containing more than one parasite. Comparison of the data on these four groups of rats shows that for the first 5 days of infection parasite numbers increased according to a geometric progression, i.e. in linear progression on a logarithmic scale. In older animals the rate of this increase was lower than that in younger rats.

In each group of infected animals, the numbers of parasites, parasitized erythrocytes, and parasitized reticulocytes were the same during the first 4 days of infection, but it should be noted that the heights of the values representing these categories were lower in older animals. In each case, the curves diverged after the 4th day, when the numbers of parasites in the blood exceeded the numbers of reticulocytes. Coincidentally, appreciable numbers of parasitized mature cells were found in the peripheral blood (table 3) and pluraparasitism increased. When the reticulocyte response to anemia became marked (8th to 10th day), the total number and percentage of parasitized mature red blood cells declined (fig. 1 to 5 and table 3), as did the percentage of erythrocytes containing more than one parasite. It is noteworthy that pluriparasitism of mature red blood cells almost never occurred, while in some cases a single reticulocyte contained in excess of 12 parasites.

Anemia varied directly with the severity of infection. In the 30- and 41-

TABLE 3.—*Percent parasitized red cells which are mature.*

Age group	Day of infection												
	3	4	5	6	7	8	9	10	11	12	13	14	15
30 days (fatalities)	0.86	2.54	43.31	21.82	7.54	3.01	3.65	2.58	1.66	0.95	0.47	0.23	0.22
41 days (survivors)	2.22	2.65	23.76	31.53	10.27	4.86	2.61	1.64	0.41	0.09	0.02	0.02	0.09
51 days		3.03	14.03	22.26	8.01	3.27	1.95	0.97	0.95	0.02	0.01	0.82	
81 days		1.25	4.00	17.08	6.15	4.94	1.69	0.76					

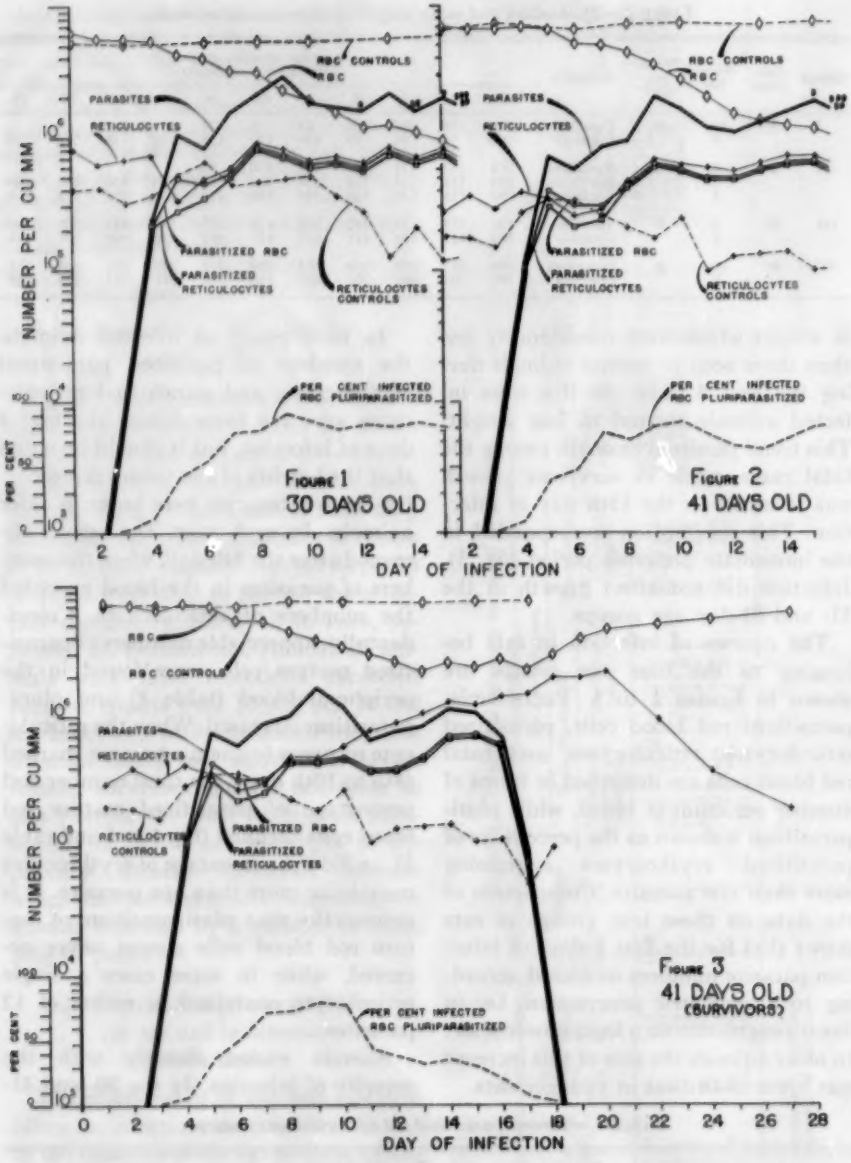

FIG. 1.—The course of infection in rats 30 days of age. D indicates the death of one rat.
FIG. 2.—The course of infection in rats 41 days of age. D indicates the death of one rat.
FIG. 3.—The course of infection in 41-day-old survivor rats.

day-old groups, where parasitemia rose most rapidly, anemia started earlier (4th to 6th day), while in the 51- and 81-day-old groups erythrocyte counts did not begin to drop until the 6th to 8th days. Reticulocyte response, in

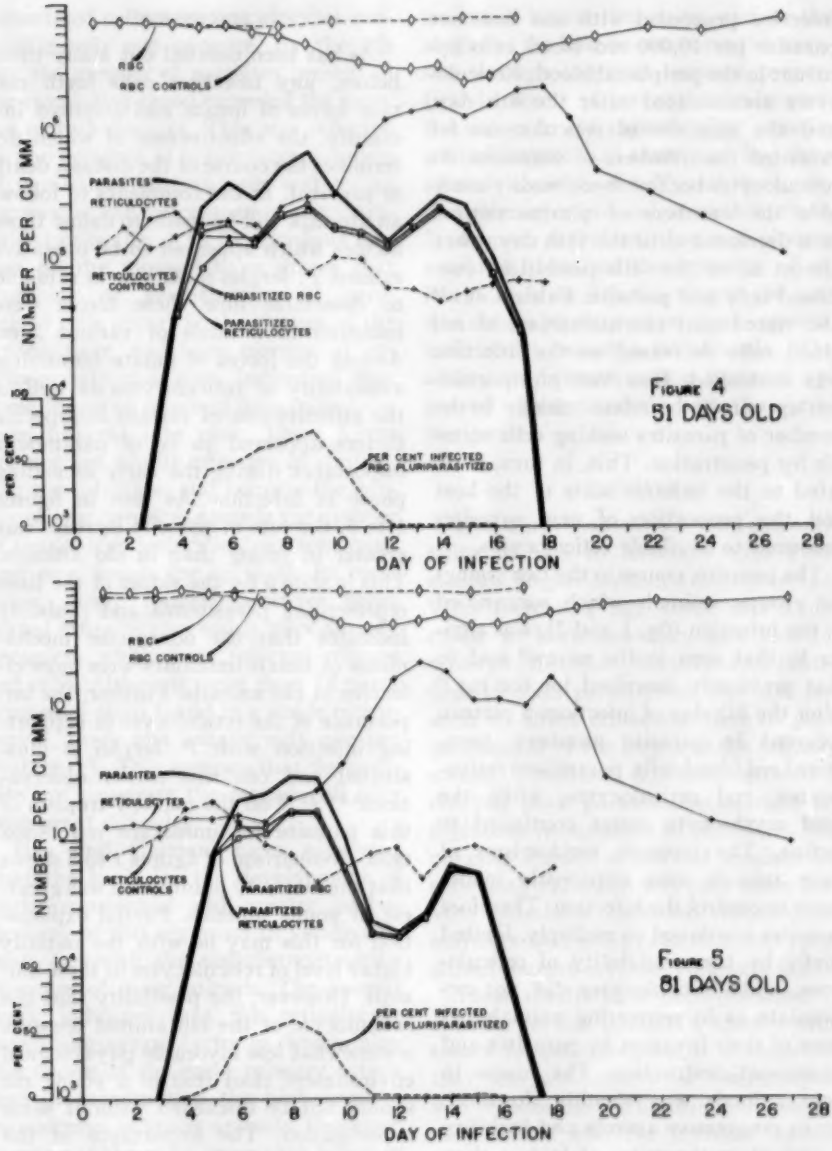

FIG. 4.—The course of infection in 51-day-old rats.
FIG. 5.—The course of infection in 81-day-old rats.

turn, directly reflected anemia. In animals which recovered (fig. 3 to 5), the infection came under control on the 8th or 9th days. Parasite numbers declined until the 12th day when a recrudescence occurred. Between the 15th and 18th days, parasites were removed from the blood stream in logarithmic fashion and

infection proceeded with less than one parasite per 10,000 red blood cells apparent in the peripheral blood. Reticulocytes accumulated after the 8th day, and the numbers of reticulocytes far exceeded the numbers of parasites. As reticulocytes became more readily available the numbers of pluriparasitized cells decreased until the 11th day, when almost all of the cells parasitized contained only one parasite. Fabiani et al[8] also noted that pluriparasitism of red blood cells decreased as the infection was controlled. However, pluriparasitism appears to be related chiefly to the number of parasites seeking cells suitable for penetration. This, in turn, is related to the immune state of the host and the proportion of new parasites produced to available reticulocytes.

The parasitic course in the two youngest groups, animals which succumbed to the infection (fig. 1 and 2), was similar to that seen in the mouse[9] and to that previously described for the rat.[10] After the 8th day of infection, a plateau occurred in parasite numbers, parasitized red blood cells, parasitized reticulocytes, and reticulocytes, while the total erythrocyte count continued to decline. The immune mechanisms of these animals were apparently inadequate to control the infection. Therefore parasites continued to multiply, limited chiefly by the availability of reticulocytes, while reticulocytes did not accumulate as in recovering animals because of their invasion by parasites and subsequent destruction. The course in these animals was steadily downhill, due to progressive anemia and inability to replace erythrocytes as fast as they were parasitized and destroyed.

8. Fabiani, G., Grellet, P., Vargues, R., Fulchiron, G. and Verain, A. 1952, Ann. Inst. Pasteur, **82**: 721–738.
9. Singer, I. 1954, J. Infect. Dis. **94**: 237–240.
10. Mercado, T. I. and Coatney, G. R. 1951, J. Nat. Malar. Soc. **10**: 359–365.

DISCUSSION

As has been pointed out many times before, any infection calls forth certain forces of innate and acquired immunity, the effectiveness of which determines the course of the disease, death or survival. In the comments to follow, an attempt will be made to define those factors which appeared to be operative against *P. berghei* infections in rats and to determine how these forces were balanced in animals of various ages. Among the forces of innate immunity, availability of reticulocytes as well as the effectiveness of certain nonspecific factors appeared to be of paramount importance during the early ascending phase of infection. As seen in figures 1 to 5, the rate of parasite increase was greater in young than in old animals. This is shown by the slopes of the lines representing parasitemia and probably indicates that the nonspecific mechanisms of innate immunity were more effective in old animals. Further, the importance of the reticulocyte in supporting infection with *P. berghei* is illustrated and confirms earlier observations[2,5–7,11–13] on the marked tropism of this parasite for immature red blood cells. Comparison of figures 1 to 5 shows that the intensity of infection was greatest in young animals. Partial explanation for this may lie with the initially higher level of reticulocytes in these animals. However, the possibility that the reticulocyte of the old animal presents a somewhat less favorable physiological environment than that of a young rat should not be discarded without some investigation. The importance of the reticulocyte in limiting the infection becomes apparent after the 5th day. Until the 4th day of infection, almost all

11. Galliard, H. 1949, Bull. Soc. path. exot. **42**: 431–433.
12. Baldi, A. 1950, Riv. di malariol. **29**: 349–356.
13. Corradetti, A. and Verloni, F. 1951, Riv. Parassit. **12**: 69–84.

parasitized cells were reticulocytes containing only one parasite. By the 5th day the number of parasites present in the circulating blood exceeded the number of reticulocytes. This was reflected by the presence of appreciable numbers of parasitized mature cells and by an increase in the percentage of pluriparasitized cells (fig. 1 to 5). Previous studies on the mouse[14] have shown that clumping or agglutination of parasitized cells occurs in the sinuses of the spleen, liver, and bone marrow at approximately this time, indicating that some forces of acquired immunity have been brought into play. These forces are not yet wholly effective in controlling the infection, and because the supply of new parasites exceeds the number of available reticulocytes, parasites must find new means for survival. In most cases, newly formed parasites penetrated reticulocytes which were already occupied, while some infected mature red cells. Although more than 12 parasites were often found in a single reticulocyte, there was usually only one per mature cell. This suggests that the parasite can penetrate the mature cell only with great difficulty.

Hsu and Geiman[2] have suggested that the basis for the development of high parasitemia and greater pathogenicity of this organism in young animals lies with the polychromatophilic response of these animals. The present work indicates that the reticulocyte was an important factor in determining the course of the early primary attack and in perpetuating the high level of parasitemia in young animals. However, it was not the most important factor in determining death or survival. Recovery from infection appeared to be based more on the effectiveness of other mechanisms of innate immunity, and on the ability of the host to overcome the parasite through acquired immune properties. In terms of percent reticulocytes, the values in old animals after parasite control approached those seen in young animals about to die. Nonetheless, parasitemia decreased below visible levels. It seemed, therefore, that reticulocyte availability limited the intensity of infection only until such a time as the host was able to produce an immune response sufficient to control parasitemia. The ability to produce such a response appeared to vary directly with the age of the rat.

14. Singer, I. 1954, J. Infect. Dis. **94**: 241–261.

SUMMARY

This study has shown that susceptibility of rats to infection with *Plasmodium berghei* depended clearly on age. With increasing age there was a decrease in both mortality rate and intensity of infection. Survival apparently depended on parasitemia. Age did not seem to affect either the time of peak parasitemia or the length of the primary attack. Fatalities, however, showed a delayed peak parasitemia followed by a plateau in numbers of parasites. The parasite showed a marked tropism for reticulocytes, and it was only when parasite numbers exceeded reticulocyte numbers that pluriparasitism and parasitized mature erythrocytes were found.

These results have been discussed in relation to the parasite's known preference for reticulocytes. It is suggested that the availability of reticulocytes was responsible for limiting parasitemia only before effective immune mechanisms became operative and controlled the infection. However, in young animals where immunity was ineffective reticulocytes continued to limit parasitemia until death of the host occurred.

ON THE MECHANISM OF THE GRAM STAIN

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A great deal of interest has been aroused in the mechanism of the Gram reaction particularly since it was found that some antibiotics exert a selective bacteriostatic action on the Gram-positive and are inactive against most of the Gram-negative organisms. Our interest in this matter resulted from a study on the antibacterial effect of highly dispersed sulfur in which a similar difference in susceptibility was found between Gram-positive and Gram-negative bacteria (Libenson et al, 1953).

During the last decade, the trend among investigators has been to dismiss the permeability theory proposed by Benians (1919) and by Burke and Barnes (1929), and to regard the positive Gram reaction as a result of a combination between the protein components of the cell wall and the crystal violet-iodine complex. Bartholomew and Umbreit (1944), Stacey (1949) and Fischer and Larose (1952) postulated that the sulfhydryl groups of the cell wall proteins play an important role in this reaction. This assumption is based mostly on the fact that iodine reacts with SH groups transforming them into -S-S- derivatives and that mercuric chloride, which according to Bartholomew and Umbreit can partially replace iodine in the Gram procedure, also reacts with SH groups. No direct experimental proof was given however for this interpretation. If this assumption is correct and the presence of free SH groups in the cell wall is necessary for a Gram-positive reaction, it should be expected that the destruction of these groups would affect the outcome of the reaction. In effect, any agent, including iodine and mercuric chloride, capable of destroying free SH groups, when applied before the actual staining process, should render the Gram-positive organisms Gram-negative. We have tested a number of SH reagents to determine their effect on the Gram reaction.

In a symposium on the surface of the Gram-positive bacteria, Stacey (1949) postulated: "I regard the cell wall as built in layers, the layer nearest to the cytoplasmic membrane being composed of basic amino acids and sulfhydryl groups, and containing the protein component of the Gram-dye retaining material." To investigate further whether the presence of protein in the cell wall is essential for the positive Gram reaction, we have studied the effect of the Gram stain on cellulose diffusion cells, used as a model, entirely free of proteins.

The solubilities of crystal violet and crystal violet-iodine complex compound in water, ethyl alcohol and acetone were determined.

EXPERIMENTAL RESULTS

Treatment with SH reagents.—Smears of 18-hour cultures of *Micrococcus pyogenes var. aureus FDA#209*, *Escherichia coli* and *Saccharomyces cerevisiae* were treated with the following solutions of SH reagents: iodine (Lugol), 0.1% mercuric chloride, 5.0% sodium iodacetate, 0.1% sodium chloromercuribenzoate, 0.1% silver nitrate and 0.1% cadmium acetate. The treatments were applied for 1 minute and for 10 minutes before using the Gram stain (Hucker's modification; dilution 1:4). Iodine was ap-

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plied also for 24 hours. To test the possibility that during the actual staining crystal violet might denature further the proteins of the cell wall and thereby set free additional SH groups which are not destroyed by the previous treatment with SH reagents, smears were also treated with a 50% solution of urea for 1 minute and 10 minutes at room temperature and at 60 C to make certain that the proteins had been completely denatured. Then the organisms were treated with the SH reagents and finally with the Gram stain. In all the experiments, control tests were performed simultaneously. The results have shown that the treatment with SH reagents did not change the Gram-staining properties of the organisms, which would indicate that if the so called Gram-complex consists of a combination between cellular proteins, crystal violet and iodine, as it is claimed, the SH groups play no role in its formation.

Effect of the Gram stain on protein-free diffusion cells.—Two cellulose diffusion cells about 5 cm long, 1.8 cm diameter and wall thickness of 0.18 mm, adapted and tied on a glass tube, were filled with saline and immersed in crystal violet solution (Gram). After approximately 5 minutes, when the saline inside the cells became slightly colored, both cells were washed with water. One of them was introduced in iodine solution (Gram) for 30 seconds. Then both cells were rinsed with water and immersed at the same time in 95% alcohol. The one treated with iodine remained colored deep blue and the other was rapidly decolorized. This experiment shows the essential role played by iodine in precipitating the crystal violet and preventing the stain from being removed rapidly from inside the cell wall by the solvent. It also shows that a Gram-positive reaction may be obtained with a protein-free cell wall.

Another experiment consisted of introducing iodine solution into a cellulose diffusion cell and immersing it in crystal violet solution, whereby a coating of crystal violet-iodine precipitate was obtained on the outside surface of the cell. No precipitate could be detected on the inner surface. This would be equivalent to a precipitate formed on a cell wall not permeable to crystal violet. Reversing the procedure, the dye solution was placed inside and the iodine solution outside the cell, a coating of precipitate was obtained on the inner surface. After discarding the liquids and rinsing thoroughly with water, both cells were filled with saline and immersed in 95% alcohol. The cell with outside coating decolorized while the other was still colored deep blue. After immersing them in safranine solution, the cells appear Gram negative-like and gram positive-like, respectively. These results would indicate that the formation of a specific protein-dye complex is not an essential factor in the mechanism of the Gram stain.

A third experiment was performed with two cellulose diffusion cells, one of them filled with saline and the other empty and with a longitudinal slit in the wall as a counterpart of a broken bacterial cell. Both were immersed in crystal violet solution (Gram) for 5 minutes. The cells were washed with water and immersed in iodine solution (Gram) for 30 seconds. After this treatment the open cell appeared considerably darker due to the adsorption of dye on both surfaces of the wall. However, after being washed with water and immersed simultaneously in alcohol or in a mixture of alcohol-acetone, the open cell decolorized while the other remained colored blue. When treated with safranine solution, the open cell appeared Gram-negative and the whole cell Gram-positive.

TABLE 1.—*Solubility in grams per 100 ml of solvent at 20 C.*

Solvent	Crystal violet	Crystal violet-iodine complex
Distilled water	1.64	Insoluble
Ethyl alcohol, 95%	92.0	0.27
Acetone	1.45	1.56
Alcohol-acetone, 1:1	70.0	0.50

Solubility of crystal violet and crystal violet-iodine complex.—The crystal violet used was obtained from the National Aniline and Chemical Company, Inc., New York, New York, labeled certification no. NC33, with a total dye content of 93%. The crystal violet-iodine complex compound was prepared by precipitating an aqueous 1% solution of crystal violet with a slight excess of iodine 0.1 *N* solution. The precipitate was filtered, washed thoroughly with distilled water and dried in a desiccator under vacuum.

Table 1 shows the solubilities of both crystal violet and crystal violet-iodine complex compound in water, ethyl alcohol, acetone and in a mixture of alcohol and acetone.

The solubility of crystal violet in ethyl alcohol is considerably at variance with the data found in literature. Our procedure to determine the solubility consisted of weighing 10 g of crystal violet in a dry Erlenmeyer flask of 50 ml capacity and adding to it 10 ml of alcohol 95%. Stoppered with a rubber stopper, it was left for 2 days at room temperature, with occasional stirring. The solution was filtered through Whatman no. 1 filter paper into a small test tube and part of the filtrate weighed rapidly to avoid evaporation of the solvent. The alcohol was evaporated on a boiling water bath and the residue dried in the oven at 105 to 110 C. The same proportions of solvent and crystal violet were used in the solubility determinations of the dye in the alcohol-acetone mixture.

DISCUSSION

In their experiments on the removal of ribonucleic acid from the surface of the Gram-positive organisms, Henry and Stacey (1946) and Bartholomew and Umbreit (1944) found that the Gram-negative "cytoskeletons" remaining after treatment with bile salts could be replated with magnesium ribonucleate back to Gram-positive. This reversal of the Gram reaction could be accomplished only when the "cytoskeletons" were maintained in a solution of formaldehyde. This was interpreted as further evidence that the presence of SH groups is necessary for the Gram-positive reaction to take place, and that formaldehyde acted in protecting these groups against oxidation. Actually the effect of formaldehyde is to destroy the SH groups instead of protecting them, as can be seen by the fact that SH compounds treated with formaldehyde will no longer give a positive reaction with sodium nitroprusside. Wald and associates (1953) have shown that formaldehyde is a strong SH reagent. Moreover, formaldehyde not only will destroy the SH groups but will also react with the amino groups of the basic proteins, which, according to Stacey, play also an important role in the Gram reaction. The last author reported that only traces of protein are removed from the Gram positive organisms with the bile salts, most of the basic proteins remaining attached to the cell "skeketon." The effect of formaldehyde in blockading the amino groups will therefore create by artefact a "cytoskeleton" entirely different from the original one.

Burke and Barnes (1929) rejected Stearns and Stearns' (1924) original theory of a chemical combination and supported Benians' (1919–20) views that the permeability of the cell wall constitutes the main factor in the Gram

reaction. Contrary to the recent Stacey's views, Burke and Barnes have concluded from their extensive investigations that if any combination is formed between crystal violet and the cellular proteins, this combination is destroyed upon the addition of iodine in the Gram-staining procedure.

In considering these two entirely opposite views, it should be pointed out that (1) since the presence of proteins and SH groups in the cell wall does not appear to be essential for the positive Gram reaction, (2) since the free amino groups of the basic proteins of the "cytoskeleton" and the SH groups are both destroyed by formaldehyde and (3) since the destruction of the amino groups by formaldehyde does not prevent the "cytoskeleton" from being replated and thereby becoming again Gram-positive, considerable doubt arises as to whether a specific combination takes place in the Gram-staining process between the cellular proteins and the dye complex, as formulated by Stacey and by Bartholomew and Umbreit. The experimental results with the Gram stain applied on broken cells give more evidence against the validity of the theory of a chemical combination. If the Gram-positive reaction depends upon the formation of a complex combination between the components of the Gram stain and the cell wall proteins, it should be expected that when disintegrated by physical means such as gentle grinding, the open cell of the Gram-positive organisms should still retain its Gram-positiveness, since gentle grinding could not change the chemical nature of the cell wall constituents. We have repeated the experiments and confirmed Burke and Barnes' results with yeast, which show that disintegration will transform the Gram-positive cell wall into Gram-negative debris. The results with closed and open cellulose diffusion cells and the inside and outside coating experiments show that the free access of the solvent to the dye-iodine complex is an essential factor in the outcome of the Gram reaction.

Benians, and Burke and Barnes postulated that the cell wall of both Gram-positive and Gram-negative organisms is permeable to crystal violet and that the cell wall of the former is not permeable to the dye-iodine complex formed within the cell. The experimental results with a protein-free diffusion cell and the low solubility of the crystal violet-iodine complex in alcohol and acetone would support the view that the positive Gram reaction consists essentially in the formation of a substantial amount of dye-iodine complex within the cell not readily removed by the solvent. On the other hand, the cell wall of the Gram-negative organisms, unlike that of the Gram-positive, would be practically impermeable to crystal violet. The organisms will appear colored after treatment with crystal violet due to the adsorption of the dye on the outer surface of the cell wall and the complex formed after treatment with iodine will be easily removed by the solvent. In this connection, it should be noted that the higher solubility of the dye-iodine complex in acetone explains Kopeloff and Beerman's (1922) findings that acetone is a more rapid decolorizer than alcohol. The difference in the uptake of crystal violet by the Gram-positive, Gram-variable and Gram-negative organisms reported by Kennedy and Barbaro (1953) and by Barbaro and Kennedy (1954) are in agreement with our findings.

SUMMARY AND CONCLUSIONS

Gram-positive and Gram-negative organisms were treated with SH reagents prior to applying the Gram stain. Protein-free cellulose diffusion cells, used

as a model, were submitted to the action of the Gram stain.

Results indicate that neither SH groups nor basic proteins play any specific role in the mechanism of the Gram reaction.

The results give support to the view that the permeability of the cell wall to crystal violet, the low solubility of the dye-iodine complex in alcohol and acetone, and the free access of the solvent to the complex constitute the main factors involved in the mechanism of the Gram stain.

REFERENCES

Barbaro, J. F. and Kennedy, E. R. 1954, A quantitative Gram reaction. J. Bact. **67**: 603–607.

Bartholomew, J. W. and Umbreit, W. W. 1944, Ribonucleic acid and the Gram stain. J. Bact. **48**: 567–578.

Benians, T. H. C. 1919, A further investigation into the principles underlying Gram's stain, with special reference to the bacterial cell membrane. J. Path. & Bact. **23**: 401–412.

Burke, V. and Barnes, M. W. 1929, The cell wall and the Gram reaction. J. Bact. **18**: 69–92.

Fischer, R. and Larose, P. 1952, Contribution on the behaviour and structure of the cytoplasmic membrane of bacteria. Canad. J. Med. Sc. **30**: 86–105.

Henry, H. and Stacey, M. 1946, Histochemistry of the Gram staining reaction for microorganisms. Proc. Roy. Soc. **133**: 391–406.

Kennedy, E. R. and Barbaro, J. F. 1953, Quantitative absorption of crystal violet. J. Bact. **65**: 678–680.

Kopeloff, N. and Beerman, P. 1922, Modified Gram stains. J. Infect. Dis. **31**: 480–482.

Libenson, L., Hadley, F. P., McIlroy, A. P., Wetzel, V. M. and Mellon, R. R. 1953, Antibacterial effect of elemental sulfur. J. Infect. Dis. **93**: 28–35.

Stacey, M. 1949, The nature of the bacterial surface. A symposium. Springfield, Ill., Charles C Thomas, p. 43.

Stearn, E. W. and Stearn, A. E. 1924, The chemical mechanism of bacterial behaviour. II. A new theory of the Gram reaction. J. Bact. **9**: 479–489.

Wald, G., Greenblatt, C. and Brown, K. P. 1953, Aldehydes as sulfhydryl reagents. Fed. Proc. 285–286.

PATHOGENESIS OF EXPERIMENTAL HISTOPLASMOSIS IN MICE

II. COMPARISON OF THE INTRAVENOUS AND INTRAPERITONEAL ROUTES OF INFECTION; COMPARISON OF THE PATHOGENICITY OF FOUR STRAINS OF *HISTOPLASMA CAPSULATUM*

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To study the pathogenesis of experimental histoplasmosis in laboratory animals, methods were developed for accurately measuring infecting dosages of the yeast phase of *Histoplasma capsulatum*.[1] In the present report the intravenous and intraperitoneal routes of infection and the pathogenicity of four strains of histoplasma are compared. For both comparisons, two measurements are used: the death producing effects of large dosages of yeast cells, and the infectivity of small numbers of yeast cells.

MATERIALS AND METHODS

Since the materials and methods used have been presented in detail,[1] they are summarized only briefly. Four strains of histoplasma were selected because of their varied histories and cultural characteristics. Strains 6617 and 6627 produce granular colonies and yeast phase suspensions contain many large aggregates of cells; strains 6622 and 6624 produce mucoid colonies and suspensions contain mostly single cells and small aggregates of cells. Yeast phase cultures were grown on slants of glucose cystine blood agar; slants were inoculated with standardized inoculums and incubated for 48 hours at 37 C.

Yeast cells were washed and diluted in saline at room temperature. Washed cells were differentially centrifuged to remove large aggregates of cells. Dosages were estimated from haemocytometer counts; the number of cells and the number of aggregates in the supernatant suspension were determined. For the cell count attached buds were counted as cells and for the aggregate count single cells as well as aggregates were counted as aggregates. The average number of cells per aggregate was determined by dividing the total cell count by the total aggregate count. Appropriate dilutions of the supernatant suspension were made for injecting mice and for inoculating flasks for colony counts; the volume of suspension for all inoculums was 0.5 ml.

The dosages for comparing the death-producing effects of strains were an estimated 2, 4 and 8×10^6 yeast cells per mouse. Groups of mice were injected in the order the dosages are given; intraperitoneal injections were done before intravenous injections. The dosages for comparing the infectivity of strains were an estimated 10 and 100 aggregates per mouse.

The number of viable aggregates for a given dosage was estimated from the average colony count of 10 or more flasks, each inoculated before mouse injections with an estimated 100 aggregates. In each experiment, flasks inoculated after a 2-hour interval with the same suspension showed 10 to 20% fewer colonies. For colony counts the inoculum was spread over the surface of 20 ml of medium solidified on the bottom of 250 ml Erlenmeyer flasks.† The flasks were incubated at 30 C and the mycelial colonies were counted.

General purpose Swiss white male mice, 5 to 6 weeks old, and weighing 18 to 20 g, were used. All cultures of mouse tissues were made on slants of modified Sabouraud's glucose agar.

EXPERIMENTS

1. Comparison of intravenous and intraperitoneal routes of infection

The death-producing effects of intravenously and intraperitoneally injected yeast cells of two strains of histoplasma

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1. Rowley, D. A. and Huber, M. 1954, Pathogenesis of experimental histoplasmosis in mice. I. Measurement of infecting dosages of the yeast phase of *Histoplasma capsulatum*. J. Infect. Dis. 96: 174–183.

† The medium contains per liter: 10 g Casamino Acids (Difco), 20 g glucose, 15 g agar, 1 mg Ca-pantothenate, and 0.5 mg biotin. The pH of the Casamino Acids solution was adjusted to 6.5 with 2*N* KOH.

TABLE 1.—*Death-producing effects of intravenously and intraperitoneally injected yeast cells of 2 strains of* H. capsulatum.

Strain of *H. capsulatum*	Estimated dosage of yeast cells per mouse			Route of infection				
				Intravenous			Intraperitoneal	
	Number of cells $\times 10^6$	Number of aggregates $\times 10^6$	Number of viable aggregates $\times 10^6$	Time 50% of mice were dead	Number of mice dead at 30 days	Number of mice dead at 60 days	Number of mice dead at 30 days	Number of mice dead at 60 days
6617	8	3.64	2.90	8 days	20/20		2/18	3/18
	4	1.82	1.45	12 days	19/19		ND	ND
	2	0.91	0.72	19 days	17/19	19/19	ND	ND
6624	2	1.25	0.91	14 days	14/18	14/18	0/20	0/20

ND = not done.

are compared in table 1. For strain 6617, the average number of yeast cells per aggregate was 2.2 cells and the number of viable aggregates per estimated 100 was 80. Twenty of 20 mice injected intravenously with 8×10^6 yeast cells per mouse were dead at 30 days and only 3 of 18 mice injected intraperitoneally with the same dosage were dead at 60 days. In the same experiment 17 of 19 mice injected with 2×10^6 yeast cells intravenously were dead at 30 days. Histoplasma was isolated from all surviving mice.

For strain 6624, the average number of yeast cells per aggregate was 1.6 cells and the number of viable aggregates per estimated 100 aggregates was 73. Fourteen of 18 mice injected intravenously with 2×10^6 yeast cells per mouse were dead at 30 days and none of 20 mice injected intraperitoneally with the same dosage was dead at 60 days. Histoplasma was isolated from 22 of 24 surviving mice. All cultures of tissues of 2 mice were contaminated.

In other experiments with strain 6617, dosages of 4 to 8×10^7 yeast cells per mouse injected intraperitoneally produced about 50% deaths within 30 days. It is evident that comparable dosages of yeast cells of 2 strains of histoplasma have greater death-producing effects when injected intravenously.

The intravenous and intraperitoneal routes of infection were compared by measuring the infectivity of small dosages of yeast cells. Each mouse was injected via one of the two routes with an estimated 100 aggregates of strain 6617. The average number of cells per aggregate was 2.2 cells and the number of viable aggregates per estimated 100 aggregates was 76. The mice were sacrificed 6 weeks after injection. Histoplasma was isolated from 20 of 20 mice injected intravenously and from 19 of 20 mice injected intraperitoneally. All cultures of tissues of one mouse were contaminated and histoplasma was not isolated from that mouse.

The intravenous route of infection has been used for most subsequent studies on the pathogenesis of experimental histoplasmosis.

2. Comparison of the death-producing effects of intravenously injected yeast cells of four strains of histoplasma

For each strain more than 10^5 yeast cells injected intravenously are necessary to produce fatal infections in mice. In preliminary experiments dosages of 5 to 10×10^5 yeast cells per mouse of strains 6617 and 6624 only occasionally killed mice in less than 30 days, while dosages of 10^7 yeast cells killed all mice in less than 30 days. On the basis of these preliminary experiments, dosages of 2, 4, and 8×10^6 yeast cells per mouse were used for comparing the death producing effects of 4 strains of histoplasma.

TABLE 2.—*Death-producing effects of intravenously injected yeast cells of 4 strains of* H. capsulatum.

Strain of *H. capsulatum*	Estimated dosage of yeast cells per mouse			Mice		Time in days 50% of mice were dead	Number of mice dead at 30 days
	Number of cells $\times 10^6$	Number of aggregates $\times 10^6$	Number of viable aggregates $\times 10^6$	Number injected	Average weight in g		
6617	8	3.80	3.04	20	18.2	13	20/20
	4	1.90	1.52	24	18.2	26	12/24
	2	0.95	0.76	23	18.3	30	12/23
6622	8	5.70	3.86	20	20	12	20/20
	4	2.85	1.93	20	20	14	20/20
	2	1.42	0.96	19	19.9	16	18/19
6624	8	5.34	3.30	22	18.7	11	22/22
	4	2.67	1.65	21	18.8	11	21/21
	2	1.33	0.82	20	18.6	13	20/20
6627	8	4.70	3.44	21	18.4	8	21/21
	4	2.35	1.72	22	18.3	11	19/22
	2	1.17	0.86	20	18.4	17	15/20

Results with these 3 dosages are presented in table 2.

The average number of cells per aggregate ranged from 1.4 cells for strain 6622 to 2.1 cells for strain 6617. The average number of viable aggregates per estimated 100 aggregates ranged from 62 for strain 6624 to 80 for strain 6617. For a given dosage of yeast cells e.g., 8×10^6 yeast cells, the estimated number of viable aggregates ranged from 3.04×10^6 aggregates for strain 6617 to 3.86×10^6 aggregates for strain 6622. For each dosage there were approximately 21% more viable aggregates for strain 6622 than for strain 6617.

The percentage of mice dead at 4 to 30 days is plotted for 2 dosages in figure 1. Only 1 of 252 mice injected with the 3 dosages died before the 7th day. With a dosage of 8×10^6 yeast cells per mouse the differences in the death producing effects of the 4 strains are small. For all strains 50% of the mice were dead between days 8 to 12 and 100% of the mice were dead between days 13 to 22. There are greater differences between strains with the lower dosage of 2×10^6 yeast cells per mouse. For strain 6624, 100% of the mice were dead by the 16th day and for strain 6617 only 52% of the mice were dead by the 30th day.

The variations found in repeated comparble experiments with strains 6617 and 6624 are presented in figure 2. It is evident that differences as great as those found between strains may be obtained with a given strain in repeated experiments. The viability of aggregates in suspension remains constant for several hours when aggregates are suspended in cold saline or cold saline with 0.1% cysteine.[1] Variations similar to those seen in figure 2 were found in subsequent experiments when suspensions were prepared in the cold. It is probable that there are only small differences in the death-producing effects of these 4 strains of histoplasma.

Individual mice vary in their response to a given dosage of yeast cells. This appears to be an inherent variability of the host not related to errors in measuring dosage or to loss of viability of the yeast cells. There was no evidence that small differences in weights of mice affected the course of infections. Howell et al have reported that male DBA line 1 mice are more susceptible to the death producing effects of intracerebrally injected yeast cells of histoplasma.[2] Pos-

2. Howell, A., Jr. and Kipkie, G. F. 1951, A comparison of the susceptibility by intracerebral inoculation of six strains of mice with male DBA line 1 mice. Am. J. Trop. Med. **33**: 33–41.

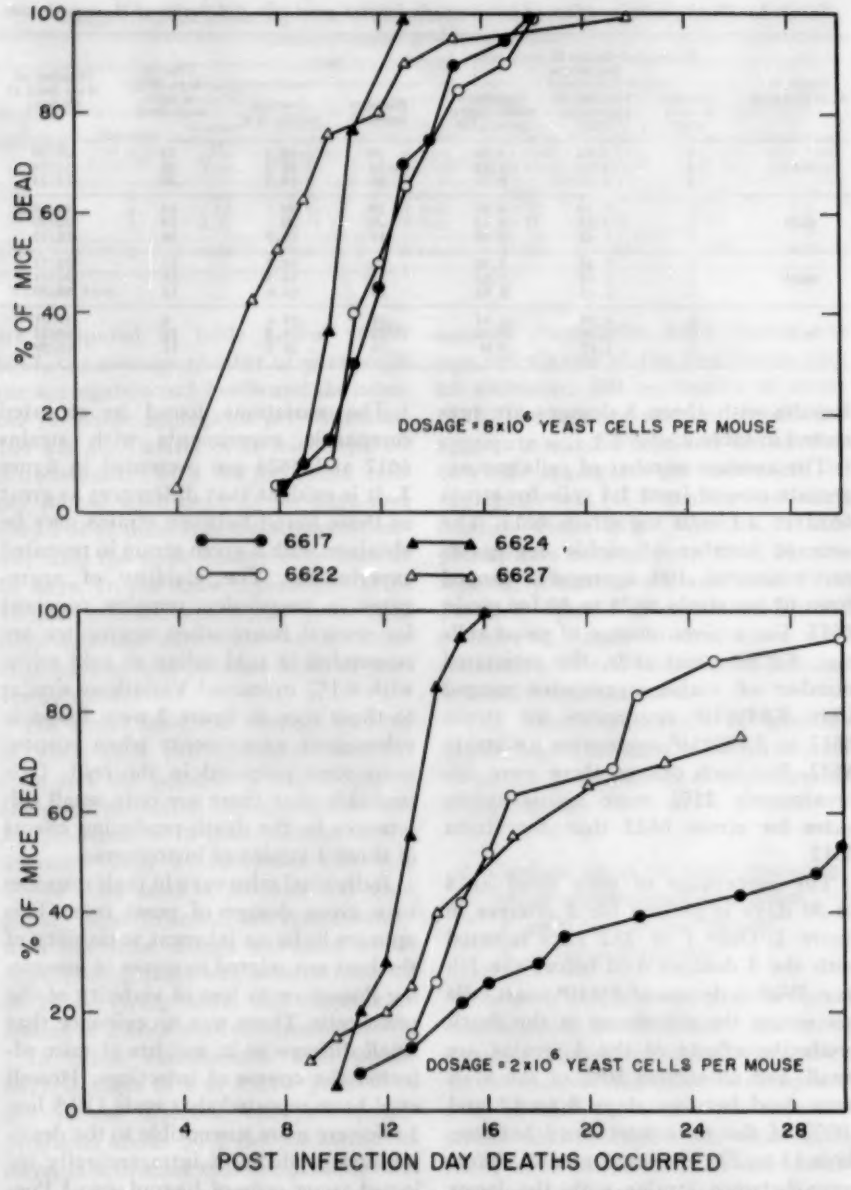

FIG. 1.—Death-producing effects of intravenously injected yeast cells of 4 strains of *H. capsulatum*.

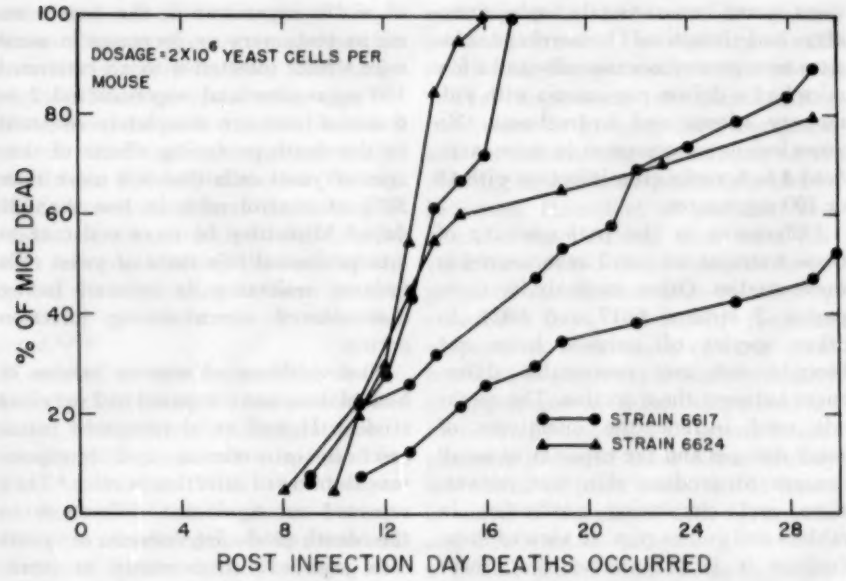

FIG. 2.—Variations in the death-producing effects of strains 6617 and 6624 in repeated comparable experiments.

sibly the use of a more highly inbred strain of mice would have reduced the variations in host responses in these experiments.

3. The infectivity of small numbers of yeast cells injected intravenously

It was previously demonstrated that for each of these 4 strains of histoplasma infections in mice were regularly produced by the intravenous injection of an estimated 100 aggregates per mouse.[1] Data were also presented which strongly suggested that 1 viable aggregate of strain 6624 injected intravenously was sufficient to infect a mouse.[1] Experiments to determine the infectivity of 1 viable aggregate for the other 3 strains have not been done. However, for each strain groups of 20 mice were injected intravenously with an estimated 10 aggregates per mouse; the approximate number of viable aggregates ranged from 4 to 9 aggregates per mouse. The mice were sacrificed 4 to 6 weeks after the intravenous injections. Histoplasma was isolated from 78 of 80 mice; all cultures of tissues of 2 mice injected with an estimated 10 aggregates of strain 6622 were sterile.

The failure to isolate histoplasma from 2 mice is difficult to explain, providing viable aggregates were injected into these mice. It seems reasonable to assume, however, that there are no important differences in the infectivity of small numbers of viable aggregates prepared from these 4 strains of histoplasma.

DISCUSSION

The pathology of nonfatal and fatal infections in mice is similar for each of the 4 strains of histoplasma. The most frequent gross lesions in fatal infec-

tions were: hepatomegaly, splenomegaly, and intestinal hemorrhage. Ascites were present occasionally and a few mice had a diffuse pneumonia with pulmonary edema and hydrothorax. No gross lesions were present in mice sacrificed 4 to 6 weeks after infection with 10 or 100 aggregates.

Differences in the pathogenicity of these 4 strains are small as measured in these studies. Other methods for comparing 2 strains, 6617 and 6624, in other species of animals have not brought out any measurable differences between these strains. The methods used include the infectivity of small dosages and the capacity of small dosages to produce skin test conversions and circulating antibodies in rabbits and guinea pigs. In view of these findings it is of interest that these strains have quite different histories and cultural characteristics.[1]

Infection with histoplasma alters the resistance of the mouse to the living organism. There is a critical range between dosages that produce chronic infections and dosages that produce overwhelming fatal infections. Dosages of 10^5 to 10^6 yeast cells injected intravenously only occasionally kill mice while dosages of 2, 4, or 8×10^6 yeast cells kill 50 to 100% of mice in less than 30 days. Mice that survive these infections for 30 days frequently continue to live as long as 6 months without clinical evidence of infection. Infections with 100 aggregates do not produce deaths in mice in periods up to 6 months. We have no evidence that mouse tissues become sterile for histoplasma after infections with 100 or more aggregates. Other types of experiments have amplified these observations. Quantitative studies on the multiplication of histoplasma in mice infected with 100 aggregates demonstrated that growth is exponential for 7 to 14 days; after 14 days the number of viable organisms in the tissues remains stationary or decreases in some mice.[3] Mice infected with an estimated 100 aggregates and superinfected 2 to 6 weeks later are completely resistant to the death-producing effects of dosages of yeast cells that kill more than 50% of control mice in less than 30 days.[3] Mice may be more resistant to intraperitoneal injections of yeast cells because resistance is induced before disseminated overwhelming infection occurs.

The virulence of various strains of histoplasma was compared in 2 previous studies. Howell et al compared intracerebral, intravenous, and intraperitoneal routes of infection in mice.[4] They reported no significant differences in the death-producing effects of yeast cells injected intravenously or intraperitoneally. With intraperitoneal injections, yeast cells suspended in mucin produced a larger percentage of deaths than similar dosages suspended in saline. The intracerebral route was superior to the other routes since fewer yeast cells produced a larger percentage of fatalities within 30 days. Marked differences in the death-producing effects of 5 strains of histoplasma were found using intracerebral injections.[5] The number of viable cells in infecting dosages was estimated from plate counts; there were more than 100-fold differences in the number of viable cells in equivalent dilutions of packed cells pre-

3. Rowley, D. A. Unpublished data.
4. Howell, A., Jr. and Kipkie, G. F. 1950, Experimental histoplasmosis. Susceptibility of male DBA line 1 mice by various routes of injection. Proc. Soc. Exper. Biol. & Med. **75**: 121–123.
5. Howell, A., Jr. and Kipkie, G. F. 1950, Studies on experimental histoplasmosis. IV. A comparison of the virulence of five strains of *Histoplasma capsulatum* by intracerebral inoculation of male DBA line 1 mice. J. Lab. & Clin. Med. **36**: 547–554.

pared from each of the 5 strains of histoplasma. No attempt was made to differentiate viable cells from viable aggregates. Small groups of mice were infected, however, with comparable numbers of viable cells or aggregates obtained from each of the strains. The organism was isolated from the tissues of almost all mice injected with viable yeast cells regardless of the route of infection, the suspending medium for the inoculum, or the strain of mice infected.[2,4–6]

Campbell et al compared the death-producing effects of 4 strains of histoplasma.[7] Mice were injected intraperitoneally with yeast cells suspended in saline or mucin. With the dosages tested, no deaths were produced when cells were suspended in saline; marked differences in the death-producing effects of 4 strains were found when cells were suspended in mucin. The viability of cells in infecting dosages was not determined, and it was reported that histoplasma was consistently isolated from mice injected with cells suspended in mucin but was rarely isolated from mice injected with cells suspended in saline.

Salvin has reported that mice (Swiss white males) are relatively resistant to intraperitoneal injections of 10, 100, or 1000 yeast cells per mouse.[8] Histoplasma was isolated from 31 of 97 mice each injected with 1000 yeast cells suspended in saline and from 47 of 97 mice each injected with the same dosage suspended in mucin. The viability of cells in infecting dosages was not determined.

The importance of standardizing conditions for growing the yeast phase and for determining the number of viable aggregates in suspensions is clearly indicated. Howell et al[6] prepared suspensions for infecting mice from cultures incubated 5 days; Campbell et al[7] prepared suspensions from cultures incubated 4 days, and Salvin[8] prepared suspensions from shake cultures incubated 48 hours. A rapid loss of viability of aggregates in saline suspension at room temperature (24 C) occurs in suspensions prepared from either heavily inoculated cultures incubated 48 hours or cultures inoculated with fewer cells and incubated for 4 days.[1]

Colony counts made on an appropriate medium give an index of the number of viable cells in a suspension only if a majority of the aggregates consist of single cells or cells with single buds. If dosages are estimated from cell counts, the number of cells per aggregate should be small; otherwise, there may be significant variations in the number of aggregates present in different suspensions containing the same number of cells. The number of viable aggregates may be more important than the number of viable cells injected, since each aggregate, though multicellular, may establish only a single focus of infection.

The intracerebral route of infection is undoubtedly superior to other routes of infection for producing deaths in mice;[4,9] however, a granulomatous meningitis is an almost constant finding.[5,6] For our studies intracerebral injections have not been used because: (1) a meningitis is produced which is rarely found

6. Howell, A., Jr. and Kipkie, G. F. 1950, Studies on experimental histoplasmosis. I. A report on intracerebral inoculations of male DBA line 1 mice. Pub. Health Rep. **65**: 722–735.
7. Campbell, C. C. and Saslow, S. 1950, Use of mucin in experimental infections of mice with *Histoplasma capsulatum*. Proc. Soc. Exper. Biol. & Med. **73**: 469–472.
8. Salvin, S. B. 1954, Cultural and Serologic studies on nonfatal histoplasmosis in mice, hamsters, and guinea pigs. J. Infect. Dis. **94**: 22–29.
9. Salvin, S. B. 1953, Immunization of mice against *Histoplasma capsulatum*. J. Immunol. **70**: 267–270.

in hosts naturally infected or in animals experimentally infected via other routes, (2) the volume of inoculum must be small, (3) the location of injections is not exact and deaths may be produced by focal brain lesions, and (4) intracerebral injections are not made easily in most other species of laboratory animals.

In our studies given dosages contain comparable numbers of small viable aggregates of yeast cells. With intravenous injections there is uniform accuracy in delivery of an inoculum; the yeast cell aggregates are cleared from the blood rapidly and almost quantitatively by the liver and spleen.[3] It is possible that testing of other strains of histoplasma with our methods might confirm previous reports of marked differences in the virulence of various strains.

The development of the experimental procedures used in these studies has made possible critical investigations of the pathogenesis of experimental histoplasmosis in laboratory animals. It is probable that these same procedures would be equally applicable to studies of the chemotherapy of experimental histoplasmosis.

SUMMARY

Methods were developed for accurate measurement of infecting dosages of the yeast phase of *Histoplasma capsulatum*. Intravenous and intraperitoneal routes of infection were compared. For 2 strains dosages of 2 to 8×10^6 yeast cells per mouse had greater death-producing effects injected intravenously. With either route infections were regularly produced with an estimated 100 aggregates of yeast cells per mouse.

Four strains of histoplasma were compared using intravenous injections. For each strain there was a critical range between dosages that produced chronic infections and dosages that produced overwhelming fatal infections. The death-producing effects of 8×10^6 yeast cells per mouse were similar for the 4 strains. There were apparent differences in the death-producing effects of the strains with a dosage of 2×10^6 yeast cells per mouse. However, differences as great as those found between strains were obtained with a single strain in repeated experiments. Infections were regularly produced with an estimated 10 aggregates of yeast cells per mouse for 3 strains; 2 of 20 mice were apparently not infected with this dosage prepared from 1 strain.

With the measurements used, no important differences in pathogenicity of 4 strains of histoplasma were found. These findings are discussed in relation to the reports of previous investigators.

THE INFLUENCE OF DURATION OF PANTOTHENATE DEPRIVATION UPON NATURAL RESISTANCE OF RATS TO A CORYNEBACTERIUM

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In previous reports[1,2] the loss of species determined natural resistance to a certain corynebacterium has been described in young rats maintained on a pantothenate-deficient diet. The present work was undertaken to study the deterioration of resistance in animals on experimental diet for varying periods of time, and to investigate suspected differences in the reaction of three strains of rats developed in this laboratory.[3]

EXPERIMENTS AND RESULTS

First experiment.—In the first experiment 16 litters, a total of 105 rats, were used. The number in each litter had been reduced to 6 or 7 at birth. Two strains were equally represented: the smaller "9B" and the medium sized "13C." One or 2 animals from each litter served as diet controls. All were housed in cages with ½-inch wire mesh bottoms, at first several to a cage, later individually. At weaning (21 days), they were all put on the same 2 diets as previously reported.[2] The deficient diet was composed of the usual purified components supplemented with ample quantities of all known vitamins except pantothenate. Calcium pantothenate was added to the diet of the controls in a concentration of 4 mg per 100 g of diet. The animals were divided into 4 groups, each group being composed of about equal numbers of the 2 strains. The 4 groups were injected with corynebacterium 197 (our strain 197, the same one used previously[2]) after 10, 20, 30 and 40 days on experiment. Injections were all intraperitoneal. Fifteen-hundredths cc of a 24-hour plain broth culture was administered. Survivors were autopsied 20 days after injection.

Several of the circumstances in the injection were controlled in part by the technique indicated below, and in part by the second experiment. Availability of animals of suitable age and strain for the first experiment was such that injections had to be made on several different days. In anticipation of this, therefore, a master culture of the organism was made on blood agar beforehand. From the master culture an ample number of subcultures on blood agar were made at the same time. For each of these subcultures several colonies from the master culture were sampled. After the subcultures had grown for 24 hours they were stored at −30 C. Before each animal injection day, one subculture was thawed, transferred to fresh blood agar, and thence into plain broth. All animals of a given litter always received inoculums from the same tube of culture. It was possible to inoculate up to 4 litters on a given day.

Results.—The results of this experiment are outlined in table 1. In order to tabulate the responses of the animals

Received for publication September 10, 1954. These data were presented in part before the American Society for Experimental Pathology, March 1954 (Federation Proc. **13**: 443).

1. Zucker, T. F. and Zucker, L. M. 1954, Proc. Soc. Exper. Biol. & Med. **85**: 517–521.
2. Seronde, J., Jr. 1954, Proc. Soc. Exper. Biol. & Med. **85**: 521–524.
3. Zucker, T. F. 1953, Rat Quality, a Consideration of Heredity, Diet and Disease, a Symposium, National Vitamin Foundation, N. Y., pp. 48–70.

TABLE 1.—*Extent of infection in rats inoculated with C197 after various periods of pantothenate deprivation.*

			10 days on diet		20 days on diet		30 days on diet		40 days on diet	
	Number of rats		3 Control	9 Experimental	2 Control	9 Experimental	4 Control	10 Experimental	3 Control	9* Experimental
9B	Survived‡	Minimal lesion	3	9	2	3	4	1	3	
		Limited infection				6		8		1
		Severe infection						1		2
	Died	Focal peritonitis								1
		Generalized peritonitis								4
		Focal and generalized peritonitis								
	Number of rats		4 Control	10† Experimental	3 Control	10† Experimental	4 Control	10* Experimental	3 Control	8 Experimental
13C	Survived‡	Minimal lesion	4	4	3		2		3	
		Limited infection		4		2	2			
		Severe infection				3				
	Died	Focal peritonitis				2		1		
		Generalized peritonitis						6		8
		Foca and generalized peritonitis				1		2		

Minimal lesion.—No gross evidence of infection; fibrous peritoneal adhesions; or single nonpurulent minute granuloma at site of injection.

Limited infection.—Purulent abscesses, either limited to the injection site, or with up to 3 additional lesions of a similar sort.

Severe infection.—Four or more purulent abscesses in the peritoneal cavity and frequently in the thoracic cavity as well. These animals also survived the arbitrary 20 day period after injection.

Focal peritonitis.—Many acute abscesses in the peritoneal cavity and often in organs. These animals and those of the 6th category lived for a week or more.

Generalized peritonitis.—Overwhelming involvement of the entire peritoneum. The pericardium and pleura were also sometimes involved. These animals sometimes showed evidence of terminal blood-stream invasion and occasionally had abscesses in organs as well. This form of infection was the most rapidly fatal, often killing in 2 or 3 days.

Focal and generalized peritonitis.—A combination of the 2 types of lesion. Evidence suggests that the latter is a terminal development.

* One animal died of intercurrent infection.
† Two animals died of intercurrent infection.
‡ Arbitrary 20-day period.

for comparative analysis, simplified categories were devised at some sacrifice of individual variation. Full justice will be done to whatever significance this variation may have when detailed description of the lesions encountered is published.[4]

It is immediately evident that susceptibility increases in both strains as the animals remain longer on their deficient diets. The previously reported[2] resistance of pantothenate-fed littermate controls is again demonstrated.

A remarkable difference in the rate of development of susceptibility is evident between the 2 strains. Thus, even after 10 days of deprivation, half of the 13C animals showed more than a minimal infection. This finding would not be significant in itself, but it has value in the context of the entire table. Only at 20 days on diet is the more resistant 9B in a corresponding state. By this time fatal infections are appearing among the 13C's. In the 30 day group all the 13C's succumbed, and all the 9B's survived, although some had more severe infection than might be inferred from the table. One 9B was still highly resistant. Forty days on deficient diet finds the 13C's highly susceptible; the longest survival was 6 days and the average was 3½ days. Autopsy findings were quite uniform. Among the 9B's this group furnishes the first fatalities.

Five 13C's died of the intercurrent disease. In 2 of these, where postmortem change was not excessive, a pseudomonas was cultured from lesions bearing no resemblance to those produced

4. Seronde, J., Jr. In preparation.

by the corynebacterium. We have occasionally encountered fatal infections with this organism in pantothenate and other deficiencies but have not yet explored their significance.

Throughout the experiment all animals were examined at frequent intervals, and their weights were recorded. Rectal temperatures taken daily on a series of them failed to reveal significant variations until the individuals were moribund. External stigmata of pantothenate deficiency were often negligible, and never severe. Varying amounts of brick-red porphyrin pigment appeared around the eyes and on the head fur in general. Internal changes notably affecting the adrenal cortexes, will be considered in a later report. Growth curves were similar to others that have been reported in pantothenate deficiency.[5] The animals gained weight in a nearly normal manner for about the first 10 days of deprivation, then their growth curves began gradually to approach the horizontal. The 2 strains were entirely comparable in this, the growth of the 13C giving no indication of its greater vulnerability to infection. Indeed susceptibility was occasionally demonstrable to some extent before deviation from the control growth pattern had developed.

Second experiment.—A second experiment was undertaken to determine the effects of any unintentional variations in dosage of organisms administered to the animals. The influence of the injected broth medium was controlled as well. And since these cultures were derived from another master culture 3 months later, the observations have some bearing on possible fluctuations in virulence. Also, data were obtained on the large 14C strain of rats[3] developed in this laboratory.

A total of 34 animals were put on the pantothenate-deficient diet at 21 days of age. Twenty-three were of the 14C strain, and 11 of the 13C strain were included for comparison. They were injected intraperitoneally with various doses of corynebacterium 197 after 30 days on diet. Survivors were killed 20 days after injection.

Using a standard series of spectrophotometer tubes, and a wavelength of 700 mμ, 24-hour broth cultures were

5. Krehl, W. A., Winters, R. W. and Schultz, R. B. 1952, Nutrition Symposium Series 5: 39–69, National Vitamin Foundation, N. Y.

TABLE 2.—*Effect of size of inoculum after 30 days of pantothenate deprivation.*

			Inoculums (all 0.15 cc)				
			Broth undiluted	Saline			
				Undiluted‡	1:5	1:25	1:125
14C	Survived	Minimal lesion				3	
		Limited infection	2	2	3	1	
		Severe infection	2	1	2	2	
	Died	Focal peritonitis					
		Generalized peritonitis	1	1	1		
		Generalized and focal peritonitis	1†	1			
13C*	Survived	Minimal lesion					1
		Limited infection		2		1	
		Severe infection			1		
	Died	Focal peritonitis		1			
		Generalized peritonitis	1		1		
		Generalized and focal peritonitis	1	1			

* One died of intercurrent disease.
† Survived.
‡ Saline suspensions of washed cells prepared from 24-hour broth culture and having the same optical density as broth culture, exclusive of color.

TABLE 3.—*Growth of deficient rats.*

Strain	Days on experiment			
	10	20	30	40
	Percent normal weight			
9B	93	75	49	46
13C	87	68	46	44
	Weight increase (W/W_I)			
9B	1.37	1.79	1.74	1.84
13C	1.55	1.87	1.86	2.06

found to have optical densities of 0.110 to 0.130. Since supernatant broth after centrifugation gives a reading of about 0.020, this leaves approximately 0.100 attributable to bacterial cells. This is an arbitrary figure naturally dependent upon the thickness of the absorbing layer. It was calibrated against the MacFarland Scale (standard $BaSO_4$ suspensions[6]), where it was found to be equivalent to 1.4. From the washed cells saline suspensions (both undiluted and various serial dilutions listed in table 2) were prepared.

Results.—The results of this experiment are summarized in table 2. First, considering dosage, it is apparent that undiluted broth and saline suspensions are equivalent. The difference between these and the 1:5 dilution is not striking. Thus this range of variation in the number of organisms administered is not particularly significant. Although numbers of animals are not great, it would appear that the response of the 13C strain in this experiment was about as previously recorded in table 1, 30-day group. Comparing this group in its entirety with the combined findings under undiluted broth and undiluted saline in table 2, the susceptibility of strain 14C is found to lie clearly between that of the 9B and the 13C.

DISCUSSION

The contrasting durations of deprivation necessary to achieve a marked susceptibility to the corynebacterium in our different strains of rat requires consideration. Examples are already known for instance where requirements for given food factors vary among strains strains within a species.[7] In order to determine the applicability of this explanation, growth data on our animals were used as an estimate of deficiency state. The use of acetylation for this purpose has been explored elsewhere.[8] Direct comparison of 9B and 13C growth curves however cannot be made because of differences in racial size. Consequently, table 3, offering 2 indirect methods, has been prepared.

By the first of these methods, tabulation of percent normal weight expected, the growth of 9B's appears slightly better than that of 13C's. By the second method, where weight increase is tabulated as the ratio of observed weight (W) to intitial weight (W_I), the opposite is inferred. Mean differences are in either case small as compared with individual values. We see, therefore, no clear indication that the strain difference in susceptibility is associated with a different deficiency status as measured by growth.

SUMMARY

Two observations have been added to our original reports.[1,2]

1. Young rats deprived of pantothenate *gradually* lose their natural species resistance to corynebacterium 197.

2. The dependence of this natural resistance upon pantothenate is influenced by the genetic constitution of the animals.

6. Kabat, E. A. and Mayer, M. M. 1948, Experimental Immunochemistry, Charles C Thomas, p. 545.

7. Fenton, P. F. and Cowgill, G. R. 1947, J. Nutrition, **34**: 273–283.

8. Zucker, L. M., Seronde, J., Jr. and Zucker, T. F. 1955, Arch. Biochem. and Biophysics, **55**: 9–14.

STUDIES IN EXPERIMENTAL MENINGITIS IN RHESUS MONKEYS

I. THE PATHOGENIC EFFECT OF VARIOUS BACTERIA RECOVERED FROM HUMAN CASES

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The original purpose of these investigations was to produce an experimental exudative meningitis, and then to determine the effect of fibrinolytic and other enzymes upon the exudate. The criteria proposed for the experimental infection were (1) that there be an abundant exudate, (2) that the degree of exudate be uniformly reproducible, and (3) that the infected animal survive long enough to allow the therapeutic effect of the enzymes to be studied.

During the course of the studies a good deal was learned about the natural history of experimental meningitis, produced by the intracisternal inoculation of various bacteria, especially in relation to such factors as mortality and degree of exudate laid down. For instance, quite different effects were produced by *Diplococcus pneumoniae*, type I, on the one hand, and *Hemophilus influenza*, type B, on the other.

When the original criteria were finally fulfilled, the suspending agent for the bacterial inoculum proved to be of crucial importance. Over a period of a year and a half, gelatin-Locke's solution was employed, but a satisfactory experimental procedure was not obtained until a strain of *Pseudonomas aeruginosa* was suspended in ground up jelled agar. By means of this technique it became possible to produce an infection in which the three criteria described above were fulfilled.

This paper will describe our experience with meningitis produced by *D. pneumoniae*, types I and III, *Streptococcus salivarius*, *Escherichia coli*, *H. influenza B*, and *Ps. aeruginosa*. The second paper in the series will deal with experiments in which enzymes were injected into the subarachnoid space.

METHODS

Monkeys.—The experimental animals were rhesus monkeys, (*Macaccus mulatta*) weighing 6 to 9 pounds, both male and female. Most of the monkeys were donated by Dr. L. H. Schmidt of the Institute for Medical Research, Christ Hospital, Cincinnati, where they had been used in an investigation of malaria chemotherapy. They had been cured of their malaria and their spleens removed. Some animals were purchased directly from a dealer.

Preparation of the bacterial inoculum.—In all the experiments the volume of the inoculum was 1 ml. In the early experiments the suspending fluid was usually Locke's solution containing 0.1% gelatin. In a few experiments using *Ps. aeruginosa* other suspending mediums were used: a simple aqueous extract of macerated monkey skeletal muscle, a similar extract of monkey liver, and yeast-beef broth. No significant differences were noted among these suspending agents in regard to the production of meningitis.

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In the later experiments nutrient agar was used as the suspending agent for the pseudomonas and the inoculum was prepared as follows. A 24-hour-old broth culture of the organism was employed. Tenfold serial dilutions were made in saline solution, except for the final dilution, which was made in 9 ml of warmed liquid 1.5% nutrient agar. This was allowed to cool and solidify in a sterile mortar. It was then ground with a sterile pestle until a smooth, semiliquid homogeneous jelly was obtained in which no individual particles could be seen. The volume at this point was 10 ml. Five ml of yeast-beef broth were then added and mixed thoroughly with the pestle to reduce the viscosity of the inoculum. One ml aliquots of this suspension were drawn into a tuberculin syringe through an 18 gauge needle. Before inoculation, the 18 gauge needle was removed and the inoculation was made through a 1½ inch #20 gauge lumbar puncture needle inserted into the cisterna magna.

It should be noted that the addition of 5 ml of yeast beef broth produced a 15-fold dilution in the final step, rather than a 10-fold dilution as in the preceding steps. The addition of the yeast beef broth reduced the percentage of agar present from 1.5% to 1%.

In order to determine whether or not the material in the mortar was of uniform composition, 1 ml aliquots were obtained before withdrawing the first inoculum, and again after all the material for inoculums had been removed. A pour plate was made from each of these two aliquots. A very close correlation in colony count was obtained between these two plates.

Bacteria employed. D. pneumoniae I.—This strain was cultured from the blood of a patient with pneumococcal pneumonia.

D. pneumoniae III.—This strain of low mouse virulence was kindly supplied by Dr. Colin M. MacLeod.

H. influenza B.—The "Keller" strain was recovered from a child with influenzal meningitis in New York and sent to us in lyophilized form by Dr. Hattie E. Alexander.

Esch. coli, type 0-07, strain ZG.—This organism was cultured from the spinal fluid of a patient who suffered two relapses of meningitis, at intervals of several weeks.

Ps. aeruginosa Kreich.—This strain was recovered from the spinal fluid and brain of a patient who died of subacute meningitis.[1] At autopsy, the brain displayed extensive thick yellow-green exudate.

Str. salivarius M.W.—This organism was recovered from the blood stream of a patient with subacute bacterial endocarditis complicating congenital heart disease.

Technique of cisternal puncture.—The animals were anesthetized with an intravenous or intraperitoneal injection of pentobarbital. Occasionally it was necessary to use open drop ether in addition. A 20-gauge lumbar puncture needle was inserted into the cisterna magna, and after the withdrawal of 1 or 2 ml of fluid, the bacterial inoculum was introduced. Subsequent cisternal punctures were repeated at appropriate intervals, sometimes daily.

Study of spinal fluid.—The following examinations were performed on each specimen of spinal fluid: white cell count, red cell count, Pandy reaction, culture, Gram stain. Some of the fluids were centrifuged and the supernatant frozen at 4 F for further studies.

Autopsies.—Autopsies were performed on animals which died as a result of the experimental infection. Of the monkeys which survived, most were sacrificed and the meninges and brain removed; a few were allowed to live. At autopsy, slices of the arachnoid and underlying brain were fixed in neutral formalin buffered with phosphate. All sections were stained with hematoxylin and eosin, some by the Feulgen technique, and some by Mallory's phosphotungstic acid hematoxylin stain. Cultures were made of any exudate present.

EXPERIMENTAL RESULTS

Meningeal exudate.—That the various microorganisms differed in the amount of exudate they elicited in the meninges is demonstrated by the data in table 1. This table was constructed from observations on autopsy material on all monkeys which died spontaneously or were sacrificed. Certain animals were permitted to survive well beyond their clinical recovery and a few animals which died within the first 48 hours after inoculation were discarded without autopsy.

The only two bacteria which regularly produced enough exudate to be desig-

1. Biehl, J. P. and Hamburger, M. 1954, Polymyxin B therapy of meningitis following procedures on central nervous system. Beneficial therapeutic effect of polymyxin B in meningitis due to organisms of the colon group. Arch. Int. Med. 93: 367–378.

TABLE 1.—*Meningeal exudate produced by different organisms.*

Microorganism	Number of monkeys	Percent of monkeys whose brains showed varying amounts of exudate			
		None	Scanty	Moderate	Profuse
Ps. aeruginosa (agar)	26	0	15	12	73
D. pneumoniae I	56	9	23	43	25
Esch. coli	10	30	20	30	20
H. influenza B	4	25	25	0	50
D. pneumoniae III	3	67	0	33	0
Ps. aeruginosa	12	75	17	8	0
Str. salivarius	2	100	0	0	0

Except for the experiments in the top line, where agar was employed as the bacterial suspending agent, and 3 of those in line 6 (*Ps. aeruginosa*), where yeast-beef broth was employed, the microorganisms were suspended in gelatin-Locke's solution.

nated as moderate or profuse were the *D. pneumoniae I* strain suspended in the gelatin-Locke's solution, and the *Ps. aeruginosa* when it was suspended in agar. The other strains were unsatisfactory from the standpoint of exudate production. The colon bacillus did elicit adequate exudate in half of the animals, but the infection was so rapidly fatal that it was not suitable for our purposes. The other strains produced meningitis irregularly. In the case of the *D. pneumoniae III*, *H. influenza B* and *Str. salivarius*, several animals given large inoculums failed to develop positive spinal fluid cultures, and were not sacrificed.

The *D. pneumoniae I* strain produced a uniformly fatal meningitis in every animal whose spinal fluid culture became positive, though a few monkeys inoculated with fewer than 1000 organisms failed to develop positive cultures. Inoculums larger than 1000 bacteria usually killed within 2 to 4 days with the production of little exudate. The most satisfactory dose was 100 to 999 organisms, but since even this dose usually killed in less than 4 days after inoculation, the strain was not ideal. The exudate, not abundant, was grayish or grayish-yellow in color. It was usually found in the sulci, sometimes over the cortex, and sometimes at the base of the brain. Microscopically it consisted of a great outpouring of polymorphonuclear leucocytes and abundant fibrin. Extracellular desoxyribonucleic acid was not demonstrable by the Feulgen technique.

The pseudomonas strain gave irregular results when the inoculum was suspended in gelatin-Locke's solution, the suspending agent used for the other microorganisms. Seventy-five percent of the animals showed no exudate at all, and those which did display any died on the second day of their disease.

The possibility that reinoculation of the same animal at short intervals with increasing numbers of organisms might produce conditions which would lead to localization of the meningeal infection was investigated in a group of 5 monkeys. The results were not encouraging. When reinoculated at 3- to 13-day intervals with progressively increasing numbers of pseudomonas organisms suspended in gelatin-Locke's, some protection against higher bacterial dosages (5 million to 90 million organisms) seemed to be provided by the previous inoculations (10 to 80,000 organisms). Three monkeys survived indefinitely after the final inoculation with 5 million organisms. One monkey died less than 24 hours after inoculation with 90 million organisms and at autopsy was found to have a moderate amount of meningeal exudate; cultures of the blood and of the exudate contained pseudomonas. The remaining monkey survived inoculation with 90 million organisms, and when sacrificed 5 days later, showed no meningeal exudate.

The employment of agar as a suspending agent for pseudomonas profoundly changed the nature of the experimental infection. Twenty-three of 26 monkeys exhibited profuse or moderate amounts of exudate. Animals sacrificed on the 5th day showed (with very

FIG. 1.—Brain of rhesus monkey infected with *Ps. aeruginosa* (Kreich strain) suspended in finely ground agar. Animal sacrificed on 6th day after inoculation.

few exceptions) thick yellow-gray-green exudate around the base of the brain and the cerebellum, sometimes extending several centimeters down the spinal cord itself. Figure 1 is a photograph of a typical example. There was rarely any exudate seen over the cortex of these brains.

Microscopically the sections showed great numbers of polymorphonuclear leucocytes, and much fibrin. A few small specks of agar could be identified as bluish amorphous material seen in hematoxylin and eosin stains. Feulgen stains demonstrated large amounts of extracellular desoxyribonucleoprotein.

In 10 experiments 1 animal was designated as a "survivor" and permitted to live. The fate of these "survivors" is shown in table 2. Three of the 10 monkeys died on the 5th day or earlier and their brains exhibited abundant exudate. Two were sacrificed on day 15 and day 24, respectively, and also displayed abundant exudate. One sacrificed on day 157 was normal. The remaining 5 survived from 10 to 60 days. Two monkeys which died of unknown causes on the 30th and 45th days, respectively, had normal meninges and negative cultures.

TABLE 2.—*Monkeys permitted to survive after infection with pseudomonas in agar.*

Monkey no.	Days lived	Bacterial inoculum	Exudate on brain	Brain culture
43	3	287	Profuse	+
37	3	287	Moderate	+
53	5	73	Profuse	
79	10	597	Slight	−
27	15	57	Profuse	+
92	30	621	None	−
75	45	533	None	
28	S 15	57	Profuse	+
49	S 24	27	Profuse	+
97	S 157	621	None	

S, sacrificed.

Microscopic sections of the exudate from the survival experiments showed a purely neutrophilic reaction with small amounts of fibrin in the 2 monkeys autopsied on day 3 (see table 2). On day 5 the exudate was still predominantly neutrophilic, but the basal layers of the leptomeninges showed evidences of early organization, with increased numbers of histiocytes, and a few plasma cells and fibroblasts. Depolymerized desoxyribonucleoprotein was present in large amounts, and there was a moderate amount of fibrin. This picture is typical of that seen in the majority of monkeys sacrificed on day 5. By the 10th day the reaction was predominantly histiocytic, with active organization proceeding. At 15 days the exudate consisted essentially of histiocytes, fibroblasts, lymphocytes and plasma cells. Occasional masses of neutrophiles were

TABLE 3.—*Mortality in experimental meningitis produced by intracisternal inoculation of common bacteria.*

Number of bacteria inoculated	*D. pneumoniae I*		*Esch. coli*		*D. pneumoniae III*		*Ps. aeruginosa*		*H. influenza B*		*Str. salivarius*	
	Number of monkeys	Percent mortality	Number of monkeys	Percent mortality	Number of monkeys	Percent mortality	Number of monkeys	Percent mortality	Number of monkeys	Percent mortality	Number of monkeys	Percent mortality
1–99	18	89	3	33	6	50	2	0	0		0	
100–999	40	95	0		0		6	17	0		4	0
1000–9999	4	100	6	67	0		1	0	3	0	0	
10,000–99,999	0		0		0		4	0	0		0	
100,000–999,999	0		0		0		1	100	0		0	
1 million–9,999,999	0		0		0		1	0	3	0	0	
10 million–99,999,999	0		0		0		2	0	0		2	0
>100 million	0		4	100	0		3	100	2	0	0	
Total	62	94	13	69	6	50	20	25	8	0	6	0

All inoculums were suspended in gelatin-Locke's solution, except a few of the pseudomonas. Some of the latter were suspended in an aqueous extract of monkey liver, an aqueous extract of monkey muscle, or yeast beef broth.

still seen. Collagen was present. Foreign body giant cells, first seen about the 10th day, were now numerous and striking. They often completely surrounded the agar fragments, forming multinucleated syncytial masses resembling the reaction seen in a gouty tophus.

Mortality in experimental meningitis produced by various bacteria.—The mortality produced by various bacteria when suspended in gelatin-Locke's solution is presented in table 3. Their virulence varied enormously, with *D. pneumoniae I* proving almost uniformly fatal and the Keller strain of *H. influenza B* failing to kill monkeys at all. In between these were the *Esch. coli* strain, which killed 69% of the animals, and the *Ps. aeruginosa* strain, which killed 25%. With most of the strains, the mortality was higher when larger bacterial inoculums were used. Also, in the fatal infections, the average day on which the monkeys died was later when the inoculum was smaller than when it was larger. Animals which were sacrificed are not included in this table.

Gelatin-Locke's solution was employed as the suspending agent in all the experiments in table 3, except in some of the pseudomonas experiments. With the latter, yeast beef broth, an aqueous extract of monkey liver, and an aqueous extract of monkey muscle were tried, but the results did not seem significantly different from those obtained with gelatin-Locke's solution.

Since most of the animals infected with pseudomonas in agar were sacrificed, the mortality data for this group are inadequate. Monkeys inoculated with 1000 to 9999 organisms died on an average of 3.5 days, but most of the monkeys given 100 to 999 survived indefinitely unless they were sacrificed.

Cerebrospinal fluid white cell counts.—Table 4 presents the mean white cell counts in the spinal fluid at various periods after inoculation.

In the group where gelatin-Locke's solution was employed as the bacterial suspending agent, by far the largest number of counts were performed on the 3rd day. There was considerable variation in the individual counts, which ranged from 66 to more than 18,000 per cmm. However, the mean counts tended to approximate one another regardless of the microorganism used or the virulence of the infection. For example, the mean white cell count on day 3 in the monkeys infected with *D. pneumoniae I* was 2210, where the mortality was 94%, whereas that with

TABLE 4.—*Cerebrospinal fluid white cell counts.*

Day of cisternal tap	Bacterial inoculum suspended in gelatin-Locke's solution												*Ps. aeruginosa* suspended in agar	
	D. pneumoniae I		*Str. salivarius*		*Esch. coli*		*H. influenza B*		*D. pneumoniae III*		*Ps. aeruginosa*			
	Average WBC	Number of monkeys	Average WBC	Number of monkeys	Average WBC	Number of monkeys	Average WBC	Number of monkeys	Average WBC	Number of monkeys	Average WBC	Number of monkeys	Average WBC	Number of monkeys
2	700	27									4307	15	35,671	39*
3	2210	31	797	8	1082	6	2956	8	290	3			10,707	4
4	3100	9									1910	10	27,594	6
5							1243	6			203	5	7,895	4
7											903	4		
8											793	9		
12							785	4						
19							122	5						
22							73	2						
29							25	3						

* This figure includes 28 animals treated with enzymes or intracisternal saline immediately following the withdrawal of spinal fluid.

the *H. influenza B* was 2956, where the mortality was zero.

It was interesting to note that in these monkeys, as well as in others inoculated with sterile solutions of one type or another,[2] there was no correlation between the height of the white count and the presence or absence of exudate in the meninges at autopsy a few days later. Counts of several thousand were common despite the absence of any visible exudate.

When agar was used as a suspending agent for *Ps. aeruginosa*, the white blood cell counts were much higher than when gelatin-Locke's solution was employed for the same organism. Counts between 20,000 and 40,000 were common, and one count was recorded at 110,000. That the agar itself was irritating to the meninges was shown by spinal fluid leucocyte counts of 21,900 and 5200, respectively, in two monkeys given intracisternal inoculations of sterile agar. When sacrificed 5 days after inoculation, the meninges of both animals were free of exudate.

Pandy reaction.—The Pandy reaction was uniformly positive in all monkeys exhibiting pleocytosis. In the vast majority of instances the reaction was recorded as 4+.

Spinal fluid cultures.—As table 5 demonstrates, the percentage of monkeys exhibiting positive spinal fluid cultures varied from zero in animals inoculated with *Str. salivarius* to 94% in those with *D. pneumoniae I* and 100% with pseudomonas when suspended in agar. In the very few instances where positive cultures were not obtained, fewer than 200 organisms had been inoculated, and the monkeys survived. In every instance where a positive culture developed the animal succumbed.

The *H. influenza B* experiments were particularly interesting. All 9 monkeys survived and 67% exhibited positive cultures. Two animals given more than 100,000,000 bacteria showed positive spinal fluid cultures through the 12th day of their disease, though they were

TABLE 5.—*Percent of positive spinal fluid cultures produced by various microorganisms.*

Microorganism	Number of monkeys	Percent positive
Ps. aeruginosa (agar)	26	100
D. pneumoniae I	64	94
Esch. coli	14	79
H. influenza B	9	67
D. pneumoniae III	5	40
Ps. aeruginosa	26	31
Str. salivarius	8	0

2. Hamburger, M. and Biehl, J. P. 1953, Some effects of injecting sterile solutions of streptokinase-streptodornase into the subarachnoid space of normal rhesus monkeys. J. Clin. Investigation, **32**: 391–393.

not at all clinically ill. In both these animals the spinal fluid cultures reverted to negative by the 22nd day. When an inoculum of between 1,000,000 and 9,999,999 organisms was employed, one monkey showed positive cultures through the 5th day, but negative cultures on the 12th and 19th days. Another displayed consistently positive cultures through day 19, but a negative one on day 29.

When inoculum of 100 to 9999 organisms of the pseudomonas strain was suspended in agar, 100% of 26 monkeys exhibited positive spinal fluid cultures.

Direct smears of spinal fluid from infected monkeys.—During most of the cisternal punctures a drop of spinal fluid was allowed to fall on a glass slide and was stained by the Gram technique. Every preparation from an infected monkey collected from the 2nd day to the subsidence of the infection contained large numbers of polymorphonuclear leucocytes.

The number of bacteria seen on these smears varied with the infecting microorganism. In the *D. pneumoniae I* infection few or no bacteria were seen on the 2nd day, but from then on they were found in large numbers in practically every instance. No phagocytosis was noted. In the *H. influenza* infections only 29% of the smears on the 2nd day exhibited gram-negative rods. None of the smears from taps after the 2nd day showed any bacteria at all.

In the pseudomonas infections in which the bacteria were suspended in agar 29% displayed positive smears on the 2nd day. Despite the persistence of positive cultures, the percentage of positive smears fell progressively, even in animals whose brains displayed considerable exudate at autopsy. Thus, on the 3rd day 11% of the smears were positive, on the 4th day 10%, and on the 5th day none.

DISCUSSION

The experiments reported in this paper demonstrate the difficulty we have encountered in producing a satisfactory experimental model of subacute meningitis wherein a suitable exudate would be produced regularly but in which death would not occur within the first few days. In producing such a model the employment of agar rather than gelatin-Locke's solution as the suspending agent for the infecting bacteria proved to be more important than any other single factor. The agar apparently played a dual role. It produced irritation of the meninges, and also seemed to hold the bacteria close to the site of inoculation.

In the gelatin-Locke's experiments, there was a pronounced parallelism between the virulence of the various strains for the monkey and for man. For example, *D. pneumoniae I* produced an almost uniformly fatal meningitis lasting from 1 to 5 days. The colon bacillus also caused an infection of high mortality. On the other hand, the strain of *Str. salivarius* from a case of bacterial endocarditis failed to infect monkeys at all. This is in keeping with the rarity of meningitis in man caused by *Str. salivarius*. The effect of the *H. influenza B* strain was also interesting in view of the well known fact that though it is a common cause of menigitis in infants and small children, it rarely infects adults. Since the monkeys employed were all relatively mature animals, it is noteworthy that the meningitis produced in these monkeys was mild and self-limited.

Bacterial dosage played a considerable role, and in this respect of course no comparison with human infection is possible. The role of dosage was particularly conspicious in the *Ps. aeruginosa* infections, where undiluted culture in gelatin-Locke's solution killed 100%

of animals but where small doses failed to infect at all unless the organism was suspended in agar.

It is of some interest to recall efforts to produce experimental meningitis during the early years of the 20th century. As far back as 1906, attempts to produce the disease in guinea pigs and monkeys were reported by Southard and Keene[3] and by Simon Flexner.[4] In these early experiments pneumococci and meningococci were employed, and various ways of introducing the organisms were investigated. Flexner put meningococci directly into the spinal canal by means of lumbar puncture. Southard and Keene infected guinea pigs through the orbit. In 1911 Martha Wollstein[5] infected mice, guinea pigs, rabbits and monkeys with influenza bacilli. Her strain was particularly virulent for monkeys, following lumbar inoculation, though she described one monkey which survived 27 days, with complete recovery. Austrian[6] in 1918 produced meningococcal meningitis in rabbits by the lumbar inoculation of meningococci.

In 1919 Ayer[7] reported an extensive investigation of meningitis following intravenous or subarachnoid injection of various bacteria. He was especially interested in finding means of lowering the barrier to passage of bacteria from the blood into the subarachnoid space. The most reliable method discovered was simple withdrawal of cerebrospinal fluid either by lumbar or cisternal puncture prior to the intravenous inoculation of bacteria. The most effective method was injection of concentrated saline solutions intravenously just before bacterial inoculation. In contrast with our own experience, Ayer felt that organisms most virulent in the human meninges showed little virulence in the subarachnoid space of his experimental animals. However, most of the experiments were carried out on cats, with a few rats, guinea pigs, one hen and one monkey. The difference between his results and those reported in this paper are probably explained by the difference in animal species employed.

A final point worthy of comment is the striking lack of correlation between the height of the white cell count in the spinal fluid and the presence of exudate in the meninges. Pleocytosis without exudate has been observed following injection of bacteria of low pathogenicity or of sterile irritating solutions.[2]

3. Southard, E. E. and Keene, C. W. 1906, A study of brain infections with the pneumococcus. J.A.M.A. **46**: 13–21.
4. Flexner, S. 1907, Experimental cerebrospinal meningitis in monkeys. J. Exper. Med. **9**: 142–167.
5. Wollstein, M. 1911, Serum treatment of influenzal meningitis. J. Exper. Med. **14**: 73–82.
6. Austrian, C. R. 1918, Experimental meningococcus meningitis. Bull. Johns Hopkins Hosp. **29**: 183–185.
7. Ayer, J. B., Weed, L. H., Wegeforth, P. and Felton, L. D. 1920, Monographs of the Rockefeller Institute for Medical Research, no. 12, (A Study of Experimental Meningitis), pp. 26–113, The Rockefeller Institute for Medical Research, New York.

SUMMARY

Meningitis has been produced experimentally in adult rhesus monkeys by the inoculation into the cisterna magna of the following strains of bacteria: *Escherichia coli*, *Pseudomonas aeruginosa* and *Hemophilus influenza B*, all from human cases of meningitis; *Diplococcus pneumoniae I* and *Streptococcus salivarius* from the blood stream of patients, and *D. pneumoniae III* from a case of human pneumonia.

D. pneumoniae I suspended in gelatin-Locke's solution produced a rapidly fatal infection associated with varying amounts of exudate in the subarachnoid space. *H. influenza B*, suspended in the same fluid, gave rise to a very mild in-

fection, with pleocytosis and positive spinal fluid cultures, but with spontaneous recovery and ultimately negative spinal fluid cultures in every animal. The effects of the other strains fell between these two extremes. The amount of exudate found in the subarachnoid space was not predictable. In general less exudate was found on the meninges of animals dying within 48 hours than in those which survived longer. Increases in the number of white cells in the spinal fluid, usually of the magnitude of 1000 to 4000 cells per cmm, and a positive Pandy reaction were observed in all animals which developed positive spinal fluid cultures.

Reproducible results, from the standpoint of exudate production, were achieved when the pseudomonas was suspended in finely ground agar instead of in gelatin-Locke's solution. Inoculation of such a suspension led to a regularly reproducible meningitis, with abundant thick, yellow-gray-green exudate around the base of the brain. Microscopically this exudate contained many polymorphonuclear leucocytes, fibrin, and extracellular desoxyribonucleic acid. With appropriate bacterial dosage, such animals lived for 5 days or more and provided an excellent experimental model for the study of therapeutic measures in meningitis.

LABORATORY EXPERIENCES WITH INFLUENZA IN BOSTON DURING THE WINTER 1949–50

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Both influenza A and influenza B viruses were isolated in various outbreaks that were studied by different workers in the winter of 1949–50 in many parts of the United States and in other countries.[1–7] In one hospital in New York, influenza B viruses were obtained from some patients and influenza A strains from others, and infections with these strains were confirmed by studies of the antibody response demonstrated in acute and convalescent serums from the patients.[3] Corresponding to the outbreaks in the United States, there was an increase in the number of reported deaths from influenza and pneumonia between the first week of March and the second week of April 1950.[8]

During the first two weeks of March 1950 in Boston, when acute respiratory infections resembling clinical influenza were prevalent, several strains of influenza A virus were isolated from throat washings of patients with such infections. However, none of the patients from whom these strains were isolated showed a characteristic antibody response in their serums to standard strains of influenza A virus, and the acute and convalescent serums of one of them showed a significant rise in titer of antibodies to strains of influenza B. A strain of influenza B, resembling other recent B strains but differing from Lee, was subsequently isolated from the original throat washing of the latter patient. In the second week of April, a patient with clinical influenza yielded from his throat washing a strain of virus resembling the influenza A prime strains that have been observed since 1947; this patient's serums showed a rise in titer of antibody characteristic of influenza A infection. The possibility that the earlier isolations represented laboratory contaminants, as suggested by Andrewes and his coworkers,[9–11]

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1. Hilleman, M. R., Mason, R. P. and Buescher, E. L. 1950, Antigenic pattern of strains of influenza A and B. Proc. Soc. Exper. Biol. & Med. **75**: 829–835.
2. Hilleman, M. R., Mason, R. P. and Rogers, N. G. 1950, Laboratory studies on the 1950 outbreak of influenza. Pub. Health Rep. **65**: 771–777.
3. Tamm, I., Kilbourne, E. D. and Horsfall, F. L., Jr. 1950, Comparison of influenza B virus strains from the 1950 epidemic with strains from earlier epidemics. Prc. Soc. Exper. Biol. & Med. **75**: 89–92.
4. Meiklejohn, G., Kempe, C. H., Thalman, W. G. and Lennette, E. H. 1952, Evaluation of monovalent influenza virus vaccines. II. Observations during an influenza A prime epidemic. Am. J. Hyg. **55**: 12–21.
5. Bozzo, A. 1952, Studies of the antigenic composition of influenza B viruses. Bull. World Health Org. **5**: 149–163.
6. Brans, L. 1952, Studies on the Antigenic Composition of Influenza Virus B Strains, with the aid of the haemagglutination inhibition technique. Stenfert Kroese, Leiden, 32 pp.
7. Magill, T. P. and Jotz, A. C. 1952, A pattern of influenza virus variation. J Bact. **64**: 619–628.
8. Davis, D. J. and Dauer, C. C. 1953, The occurrence of influenza in the United States, 1952–53. Pub. Health Rept. **68**: 1141–1146.
9. Andrewes, C. H. and Glover, R. E. 1944, Influenza "A" outbreak of October–December 1943. Lancet, **6**: 104–105.

was considered, and the identification of those strains as very similar to the laboratory PR8 strain seemed consistent with that possibility. The experiences with the isolation and classification of the strains of influenza virus during the winter of 1949–50 and studies of the serologic response of patients observed at that time are reported in this paper.

Patients studied.—Specimens of lungs from 2 patients with acutely fatal hemorrhagic pneumonia were submitted to this laboratory for viral isolation, one on December 15, 1949 from the Children's Medical Center and the other in mid-January from an autopsy done at this hospital. These lungs and throat washings obtained from 23 other patients during the acute febrile stage of illnesses resembling clinical influenza between December 26 and May 24 were available for this study. Acute and convalescent phase serums were also available from these 23 patients and from 9 additional patients; the acute phase serums from 3 of the latter were obtained in mid-November 1949 and the rest in January 1950. The patients from whom throat washings were obtained included 5 members of the staff of this laboratory and the wife of a sixth, and also 3 interns at this hospital and the wife of one of them; the others were adult patients admitted to the medical wards. The 5 members of the laboratory staff all collected and stored throat washings from various patients, but only one of them was concerned with the actual isolations of viruses; 2 others, however, were engaged in different studies with the PR8 strain at the same time.

Methods and materials.—The methods used in the isolation of influenza viruses, the in vitro serologic tests, the preparation of rabbit antiserums and the egg neutralization tests were similar to those employed in previous studies in this laboratory.[10–14] As in those studies, the antigenic comparisons of the strains were done with rabbit antiserums by complete cross-neutralization tests in embryonated eggs. All antibody titers are expressed as the reciprocal of the initial dilution of serum. Antigenic ratios were computed from the results of the cross-neutralization tests using the formula $R = \sqrt{r_1 \times r_2}$, as recommended by Chu, Andrewes and Gledhill[15] and by Hilleman and Horsfall.[16] In this formula r_1 and r_2 represent the heterologous titer divided by the homologous titer for viruses 1 and 2, respectively. For convenience, the ratios are expressed as percent, i.e., as $R \times 100$.

RESULTS

Isolations of virus.—The results of all the attempts to isolate influenza viruses from the lungs and throat washings are shown in table 1. Results were considered negative for influenza virus when neither amniotic nor allantoic fluids of any inoculated eggs agglutinated chicken erythrocytes after 2 or more amniotic passages, using 6 or 8 eggs for each passage. Negative results were obtained on 2 to 4 amniotic passages of each of the 3 specimens of pneumonic lung from the 2 fatal cases, and on 2 or 3 amniotic passages of the throat washings of 15 other patients; in 4 of these 15 patients similarly negative results were obtained with the same washings in a subsequent attempt. The

10. Andrewes, C. H., Glover, R. E., Himmelweit, F. and Smith, W. 1944, Influenza virus as a laboratory contaminant. Brit. J. Exper. Path. **35**: 130–133.
11. Isaacs, A. and Andrewes, C. H. 1951, The spread of influenza: evidence from 1950–51. Brit. M. J. **2**: 921–927.
12. Barnes, M. W., Morgan, H. R. and Finland, M. 1948, Isolation and identification of influenza viruses during the epidemic of December 1945. J. Lab. & Clin. Med. **33**: 309–318.
13. Morgan, H. R., Barnes, M. W. and Finland, M. 1948, Antigenic differences among influenza A viruses, including serologic responses of patients. J. Lab. & Clin. Med. **33**: 1212–1219.
14. Finland, M. and Barnes, M. W. 1955, Influenza B in Boston, 1952. Isolations of virus and serologic studies in patients, including a study of antigenic differences among influenza B viruses isolated in Boston from 1945 to 1952. Am. J. Hyg. **61**: 24–39.
15. Chu, C. M., Andrewes, C. H. and Gledhill, A. W. 1950, Influenza in 1948–1949. Bull. World Health Org. **3**: 187–214.
16. Hilleman, M. R. and Horsfall, F. L., Jr. 1952, Comparison of the antigenic patterns of influenza A virus strains determined by *in ovo* neutralization and hemagglutination-inhibition. J. Immunol. **69**: 343–356.

TABLE 1.—*Results of attempts to isolate influenza viruses from materials obtained in Boston, December 1949 through May 1950.*

Patient	Throat washing*			Patient's serologic response‡
	Date obtained	First egg inoculation	Result†	
A171	Dec. 15	Dec. 16	Am_5-Neg.	—
A47R	Jan. 18	Jan. 19	Am_5-Neg.	—
A47L	Jan. 18	Jan. 19	Am_5-Neg.	—
MF	Dec. 26	Dec. 30	Am_5-Neg.	O
VO	Dec. 27	Dec. 27	Am_5-Neg.	O
CW	Jan. 16	Jan. 30	Am_5-Neg.	O
TH	Jan. 17	Feb. 10	Am_5-Neg.	O
RW	Feb. 28	Mar. 3	Am_1-Pos. A	A?
		Mar. 14	Al_5-Pos. A	
MA	Feb. 28	Mar. 3	Am_1-Pos. A	A?
		Mar. 14	Al_5-Neg.	
		June '54	Am_1-Pos. A	
JA	Mar. 1	Mar. 3	Am_1-Pos. A	A?
		Mar. 14	Al_5-Neg.	
DP	Mar. 7	Mar. 10	Am_1-Pos. A	O
OB	Mar. 9	Mar. 10	Am_1-Pos. A	A?
JK	Mar. 10	Mar. 11	Am_1-Pos. A	O
Fs	Mar. 11	Mar. 14	Am_1-Pos. A	B
		June '54	Am_1-Pos. B	
	Mar. 12	Oct. '50	Am_1-Pos. A	
		June '54	Am_5-Neg.	
MJ	Mar. 13	Mar. 14	Am_5-Neg.	O
		July 7	Am_5-Neg.	
MB	Mar. 18	Mar. 24	Am_5-Neg.	O
		Apr. 10	Am_5-Neg.	
EK	Mar. 18	Mar. 24	Am_5-Neg.	O
		Apr. 10	Am_5-Neg.	
VS	Mar. 22	Apr. 1	Am_5-Neg.	A
		Oct. 5	Am_5-Neg.	
AD	Mar. 26	Apr. 1	Am_5-Neg.	O
AO	Mar. 27	Apr. 1	Am_5-Neg.	O
JB	Apr. 4	Apr. 24	Am_5-Neg.	O
VD	Apr. 9	Apr. 24	Am_5-Neg.	—
JM	Apr. 13	Apr. 24	Am_1-Pos. A	A
ES	Apr. 24	July 7	Am_5-Neg.	B
MM	May 24	May 24	Am_5-Neg.	O
AJ	May 24	May 24	Am_5-Neg.	O

* A171 and A47 (R&L) are lungs from patients who died with hemorrhagic pneumonia at the Children's Hospital and at this hospital, respectively (R = right lung, L = left lung).

† Neg. = negative; Pos. = positive for influenza A or B virus. Am_5 = amniotic passage, 5 times; Al_5 = allantoic passage, 5 times. The last negative and first positive passages only are indicated.

‡ A or B = fourfold or greater increase in titer with at least one strain of influenza A or B, respectively; ? = of doubtful significance. Details are given in text and tables. O = no significant rise in titer in any of the tests or with any of the viruses; — = not done.

washings from the 5 members of the staff of this laboratory and from the wife of one of them all yielded negative results.

Influenza viruses were isolated from 8 patients; in each instance the presence of virus was demonstrated on the first attempt following the initial amniotic inoculation. Two of the positive throat washings were from "medical" interns (MA and JM); one was from the wife of one of these interns (JA), a fourth was obtained from a member of the surgical house staff (RW) and the remaining 4 strains were from ward patients. All subsequent egg passages of these strains were made allantoically and the allantoic fluids of the 15th or 16th such passage of each of these original isolations was used for the preparation of rabbit antiserums for the egg neutralization tests in the antigenic comparisons.

The viruses were demonstrated in both amniotic and allantoic fluids after the original amniotic inoculations in 6 instances, but the first allantoic fluid failed to show the presence of virus in the other 2 (from patients RW and JM), although subsequent allantoic passage of the amniotic fluid yielded virus in high titer in every instance. The hemagglutination of chicken cells by the allantoic fluid of the first allantoic passage of each of these 8 strains was inhibited by anti-PR8 rabbit serum but not by anti-Lee serum.

When acute and convalescent serums of the first 3 patients who yielded influenza viruses were tested for antihemagglutinins with standard strains of influenza A and B viruses, all failed to show any rises in titer. Since the egg passages of the throat washings of these 3 patients were all started on the same day, the possibility of laboratory contamination was considered. Since the laboratory strains were already adapted to allantoic passage, the original washings from these 3 patients (RW, MA and JA) were inoculated allantoically. The allantoic fluid of the first such allantoic passage failed to show any hemagglutination in each instance, but that of the second allantoic passage of one of them (RW) did show hemagglutination and this was inhibited by anti-PR8 rabbit serum. The washings of the other 2 patients failed to yield evidence of virus after 3 successive allantoic passages. Amniotic inoculation of some of the same throat washing from

TABLE 2.—*Results of serologic tests with acute and convalescent serums of patients JM and Fz.*

Virus	Patient JM			Patient Fz		
	HI	CF	EN	HI	CF	EN
PR8	8/128	8/512	—	16/16	8/4	—
FM1	32/256	4/128	—	64/64	4/4	—
JM	16/128	4/128	<4/40	—	—	<4/4
RW	8/128	—	<4/25	—	—	<4/<4
JK	—/64	—	—	16/8	—	—
Weiss	—	—	<4/64	—	—	<4/4
Fz1	—	—	—	16/8	4/4	—
Fz2	—/16	—	—	<4/32	—	—
Lee	8/4	4/4	—	8/32	4/64	<4/40
Bon	—	8/8	—	<4/64	8/64	<4/51

Titers are expressed as reciprocals of initial serum dilution; acute/convalescent. — =not done. HI =hemagglutination-inhibition; CF =complement fixation; EN =egg neutralization.

one of the latter patients (MA) done 4 years later yielded a hemagglutinating agent in the first amniotic fluid and this was identified as influenza A in the allantoic fluid after 3 additional allantoic passages; this virus was also neutralized in ovo by antiserum prepared against the virus originally isolated from the same washing.

Further inconsistencies were noted when the acute and convalescent serums of the remaining 5 patients whose throat washings yielded influenza A viruses were tested for antihemagglutinins with the stock strains PR8, FM1, Lee and Bon. Three of these patients failed to show a rise in titer of their serums to any of these strains, one (JM) showed a marked rise in titer to both PR8 and FM1 but not to Lee and the serums of the remaining patient (Fz) showed a substantial rise in titer to both Lee and Bon but not with PR8 or FM1. In the case of Fz, throat washings that had been obtained on successive days were available. When, several months later, the second washing was inoculated into eggs, it too yielded influenza A virus. Four years later both throat washings were again used (on separate occasions 10 days apart) in further attempts, and this time the first washing yielded a virus that resembled influenza B and the second washing failed to yield virus on 2 amniotic passages.

Results of serologic tests in patients.—The acute and convalescent serums of the 8 patients from whom viruses were isolated were subjected to numerous serologic tests with "standard" influenza A and B viruses and with the strains isolated from some of these patients. Serums of the remaining patients from whom viruses could not be isolated and from those of other patients from whom throat washings were not obtained were tested only for agglutination-inhibition with the PR8 and FM1 strains of influenza A, the Lee strain of influenza B and with the strain isolated from one of the patients (OB). All the results obtained with the serums of the 2 patients JM and Fz are shown in table 2, and all other results that might be considered positive in the remaining patients are shown in table 3.

From table 2 it is seen that the tests done on the serums of JM with any of the influenza A viruses, including the patient's own strain and one isolated from another patient, all showed substantial increases in titer, but no such rises in titer were demonstrated in these serums with any of the influenza B strains.* The serums of Fz, on the other hand, yielded significant increases in titer only with influenza B strains, including the patient's own strain Fz 2,

* Unfortunately, some of these results are incomplete because the supply of acute phase serum had already been exhausted when some of these tests were done.

TABLE 3.—*Summary of positive serologic tests with influenza viruses in acute and convalescent serums of patients other than JM and Fz.*

Patient	Date of acute serum	Positive result			Tests done with acute and convalescent serums of these patients
		Virus	Test*	Result†	
JA	Mar. 1	JM	HI	<4/8	HI with PR8, FM1, Lee, Bon, JM, JA, MA, OB, JK, RW and homologous.
MA	Mar. 1	JA	HI	32/128	
RW	Feb. 28	JM	HI	4/16	CF with PR8, FM1, Lee, Bon and homologous.
OB	Mar. 9	OB	CF	<4/8	EN with RW, Weiss, Lee, JM and homologous.
MD	Nov. 14	FM1	HI	4/32	HI only—with PR8, FM1, Lee and OB.
VS‡	Mar. 21	PR8	HI	8/32	
ES	Apr. 27	Lee	HI	16/64	

* HI = hemagglutination-inhibition; CF = complement fixations. EN = egg neutralisation.
† Titers expressed as reciprocal of initial dilution of serum (acute/convalescent).
Influenza A viruses were isolated from the throat washings in the first 4 patients; throat washings were not available in MD and those of VS and ES yielded negative results (see table one).
‡ Titers obtained in this patient's serums by HI with FM1 were 64/128.

but not with any of the influenza A strains, including Fz 1, the first strain that was isolated from the same throat washing of this patient.

From table three it is seen that the results in nearly all of the remaining patients could not be considered as unequivocally indicating influenza viral infections. In only one patient, MD, was more than a 4-fold rise in titer against any strain demonstrated; in this patient, who was ill in mid-November, there was an 8-fold rise in titer of antihemagglutinins for FM1, but no rise in titer against PR8. This may represent a specific response to infection with a strain of influenza virus resembling FM1 but distinct from PR8. Fourfold rises in titer of antihemagglutinins for standard strains were also demonstrated in the serums of 2 other patients, in one with PR8, suggesting an influenza A infection, and the other with the Lee strain, suggesting an influenza B infection. In these 2 patients throat washings obtained at the time of the acute phase serum failed to yield any influenza virus.

The results obtained in the 4 patients (JA, MA, RW and OB) from whose throat washings influenza viruses were isolated can be considered positive only in that they represent 4-fold rises in titer, but they are probably not significant. In each instance the "positive" result was obtained in a single test with one of the strains isolated during the same season, but other tests with the same and other strains yielded no such rises in titer. In the other 2 patients (JK and DP) from whom viruses were isolated, no increases in titer were demonstrated in any of these tests. It is also interesting to note that no positive serologic results were obtained in any of the laboratory staff.

Antigenic comparison of influenza A strains.—Complete cross-neutralization tests in embryonated eggs were carried out with the following viruses and their antiserums: (a) the first strains obtained from the throat washings of the 8 patients studied in 1950, (b) 2 strains isolated in this hospital in 1947 and 1949[13] and (c) the 3 "standard" strains PR8, Weiss and FM1. Allantoic fluids of the 16th to 18th egg passage were used in the case of the strains isolated in this laboratory, and the standard strains had received varying numbers of allantoic passages after they were received. All the rabbit antiserums were tested simultaneously with a single virus and the number of infective doses (ID_{50}) of the inoculum was determined at the same time. The results are shown in the upper part of table 4. Antigenic ratios calculated from these results and expressed in percent are shown in the lower part of the same table.

TABLE 4.—*In ovo cross-neutralization of influenza A viruses.*

Virus	Date of washing	Inoculum ID_{50}	Titers of immune rabbit antiserums*													
			Fz1	JA	MA	RW	OB	JK	DP	JM	Coop	EB	DW	FM1	Weiss	PR8
Fz1	Mar. 11, 1950	693	*512*	1630	1420	90	512	8192	2048	<4	<4	<4	<4	<4	<4	1630
JA	Mar. 1, 1950	667	256	*1024*	256	161	512	817	1024	<4	<4	<4	<4	<4	10	168
MA	Mar. 1, 1950	667	512	406	*512*	256	161	2581	1024	<4	<4	<4	<4	<4	<4	720
RW	Feb. 28, 1950	333	406	256	128	*128*	256	5160	1630	<4	<4	<4	<4	<4	16	348
OB	Mar. 9, 1950	1,050	816	221	128	102	*128*	8192	2900	6	<4	<4	<4	<4	23	256
JK	Mar. 10, 1950	167	646	2048	649	162	512	*3260*	2545	<4	<4	<4	<4	<4	16	434
DP	Mar. 7, 1950	80	1024	2048	646	128	512	8192	*2048*	<4	<4	<4	<4	<4	64	210
JM	Apr. 13, 1950	1,053	<4	<4	<4	<4	<4	<4	<4	*1630*	512	13	<4	<4	<4	<4
Coop	Mar. 1949	33	<4	<4	<4	<4	<4	<4	<4	1630	*1630*	16	5	11	<4	<4
EB	Mar. 1947	105	<4	<4	<4	<4	<4	<4	<4	1024	512	*2580*	32	147	<4	<4
DW	Mar. 1947	3,333	<4	<4	<4	<4	<4	<4	<4	32	64	27	*256*	<4	<4	<4
FM1	1947	10,500	<4	<4	<4	<4	<4	<4	<4	2048	4096	128	40	*256*	<4	<4
Weiss	1943	1,000	32	32	10	64	26	64	40	<16	4	<4	11	<4	*1630*	128
PR8	1934	4,933	646	512	162	256	4096	2545	<4	<4	<4	<4	<4	<4	<4	*256*
			Titer-ratios calculated from these values and expressed in percent (R×100)													
Fz1			100													
JA			59	100												
MA			167	45	100											
RW			74	56	71	100										
OB			246	93	56	126	100									
JK			178	71	100	141	316	100								
DP			141	100	79	89	238	177	100							
JM			<0.4	<1	<0.4	<0.3	<0.3	<0.2	<0.2	100						
Coop			<0.4	<0.1	<0.4	<0.3	<0.3	<0.2	<0.2	56	100					
EB			<0.4	<0.1	<0.4	<0.2	<0.2	<0.1	<0.1	6	4	100				
DW			<1	<0.8	<1	<2	<2	<0.2	<0.6	<2	3	4	100			
FM1			<1	<0.8	<1	<2	<2	<0.2	<0.6	<14	<33	17	<5	100		
Weiss			<1	1	<0.7	7	5	1	3	<0.2	<0.2	<0.2	<1	<0.6	100	
PR8			<0.6	57	167	130	141	146	102	<0.6	<0.6	<0.5	<2	<2	<3	100

* Normal (pre-immunization) serum was tested simultaneously with homologous immune serum, the titer was <4 in every instance. Homologous titers are in italics. All titers are expressed as the reciprocal of the initial dilution of serum at the 50% endpoint as determined by the method of Reed and Muench.[17]

TABLE 5.—*Identification of 5 strains of influenza virus isolated in 1950 by hemagglutination-inhibition with chicken antiserums*

Chicken antiserum	Strain of virus					
	Homologous	MA1	MA2	JM	Fz1	Fz2
PR8	512	>1024	>1024	<4	512	<4
Cuppett-50	128	<4	<4	512	<4	<4
Lee	128	<4	<4	<4	<4	<4
IB1-50	8	<4	<4	<4	<4	16
Neb. A-53	16	<4	<4	<4	<4	<4

Titers expressed as reciprocals of initial dilution of serum. Strains MA1 and MA2 were isolated from the same washing on March 3, 1950 and in June 1954, respectively. Fz1 and Fz2 were isolated from the washing collected March 11 and inoculated March 14, 1950 and June 1954, respectively (see table one). The chicken antiserums and the corresponding viruses were supplied by Dr. M. M. Sigel.

From table 4 it is seen that, with the exception of JM, all of the 1950 strains included in this experiment closely resemble PR8 and differ sharply from JM and from the other A strains. Strain JM, in turn, resembles most closely the strain Coop that was isolated here in 1949 and is clearly different from the other strains, although showing some relationship to EB, isolated here in 1947, and to FM1 that was isolated elsewhere in the same year. The distinctive antigenic characters of strains Coop, EB and DW, and their relationship to FM1 were previously demonstrated[13] and are here confirmed.

Identification of strains by use of chicken antiserums.—After this study was completed a small supply of chicken antiserums against 5 characteristic strains was received. They were prepared for the World Influenza Program by Dr. M. M. Sigel. Agglutination-inhibition tests were carried out with these antiserums and 5 of the strains isolated here in 1950; 2 of these were separate isolations from the same washings of patient MA, 2 were the separate isolations from the first throat washing of patient Fz and the fifth was strain JM. The results are shown in table five, and the titers obtained with the homologous strains tested at the same time are also included in this table. These results may be interpreted as indicating that both of the MA strains and the first strain isolated from Fz were similar to PR8; the strain obtained from JM was similar to Cuppett, and the second strain isolated from the first throat washing of Fz was similar to IB1/50. The Cuppett strain was isolated at Fort Warren and is related to the so-called A prime strains of influenza virus that have been isolated in 1947 and thereafter;[7] IB1/50 was isolated in New York and is a B strain that is antigenically distinct from Lee[3] but related to other B strains that have been isolated since 1947.[7]

DISCUSSION

The importance of considering as contaminants certain strains of influenza virus that are isolated in laboratores where work with influenza viruses is being carried out was first emphasized by Andrewes and his coworkers in 1944.[9,10] They recovered 2 strains of virus in 1943 which were identical with the stock laboratory strain PR8 and found them to be mouse virulent, whereas the strains they had recently isolated in eggs were not. One of these strains was isolated after no work had been done in the laboratory for 17 days.[9] They also described 14 incidents in 10 laboratories in which they suspected laboratory "pick-ups" of influenza viruses. Most of the suspected strains resembled the WS strain (isolated in England in 1933), which was then the most widely used strain in European laboratories.[10] More recently the PR8 strain has been most universally used. A few strains resembling PR8 were submitted to the World Influenza Center during 1950–51; these were also suspected of being laboratory pick-ups and the PR8 and WS-type strains are considered by the workers at

that center to have disappeared completely.[11] Certain criteria were offered for differentiating the laboratory strains.[10,11] Other suspected laboratory pick-ups of strains of influenza virus were noted by Magill and Jotz.[7]

The detection of laboratory contamination with influenza virus and determination of the manner in which it occurs is difficult. The PR8 strain used in this laboratory at the time of the present study was well adapted to eggs. It was first considered that the failure to detect virus on the first allantoic inoculation in 3 of the suspected throat washings and on 3 successive allantoic passages in 2 of them, consituted evidence against the 3 influenza A strains originally isolated from these washings being laboratory contaminants. However, most of the evidence, particularly the failure to demonstrate a rise in influenza A antibodies in 7 of the patients and the subsequent isolation and identification of an influenza B virus from one of these same washings, the latter corresponding to the rise in influenza B antibody titer, must be taken as more evidence that the PR8-like strains were indeed contaminants. Some, but not all, of the contamination probably occurred during the collection and storage of the throat washings. It is interesting, therefore, that none of the PR8-like strains were isolated from any of the laboratory workers and that none of these workers developed antibodies to influenza A or B.

The total experience in this laboratory during the winter of 1949–50 suggests that most of the acute respiratory infections suspected of being clinical influenza during that season were probably not due to any of the recognized strains of influenza virus. On the other hand, a single strain of influenza A virus was isolated which most closely resembles another strain, Coop, isolated in this laboratory in 1949 and also reacts with chicken antiserum against a 1950 strain of influenza A but not with similar chicken serums prepared against PR8 or a 1953 strain of influenza A. In addition, a strain of influenza B was isolated which resembles another B strain isolated elsewhere during the same year, but differing from the Lee strain. The patients from whom these 2 strains were obtained each developed an increase in antibodies to influenza viruses of the homologous type.

The antigenic comparison of strain JM and the other strains of influenza A viruses, including those isolated in this laboratory in 1947 and 1949, also confirm the findings of other workers that there have been progressive changes in the antigenic pattern of influenza A viruses isolated in successive years;[1,2,7,13,15,16,18,19] similar changes have also been noted in strains of influenza B viruses isolated here[14] and elsewhere.[1,3,5–7,20] However, it has generally not been possible to differentiate strains within the influenza A group or within the influenza B group by the use of convalescent serums of adult patients, although that has sometimes been possible in the convalescent serums of children;[13,21–23] unfortunately, studies were not made in children during this season.

17. Reed, L. J. and Muench, H. 1938, A simplified method of estimating fifty percent endpoints. Am. J. Hyg. **27**: 493–497.
18. Hirst, G. K. 1947, Comparisons of influenza virus strains from three epidemics. J. Exper. Med. **86**: 367–381.
19. Van der Veen, J. and Mulder, J. 1950, Studies on the antigenic composition of human influenza virus A strains. Stenfert Kroese, Leiden, 65 pp.
20. Jordan, W. S., Jr. and Gaylin, S. D. 1953, The antigenic variation of influenza B viruses. Demonstration of an antigenic spectrum by use of titer ratios. J. Immunol. **70**: 393–399.
21. Burnet, F. M. and Stone, J. D., 1946, Serological response to influenza B infection in

SUMMARY AND CONCLUSIONS

The experiences with the isolation of influenza viruses and serologic tests for influenza viral antibodies in patients suspected of having clinical influenza during the winter of 1949–50 have been presented. Several strains of influenza A virus were isolated from throat washings of patients; all but one of them were shown to be antigenically similar to PR8 and were obtained from patients whose serums failed to demonstrate significant increases in antibodies to any of the standard influenza A strains or to their own strains. These strains were, therefore, considered to be laboratory contaminants.

One strain of influenza A and one of influenza B virus were obtained from patients whose serums showed considerable increases in titer to all strains of the homologous type with which they were tested, but not to strains of the heterologous type. Studies of these strains confirmed the observations of other workers on the changing antigenic pattern of influenza viruses.

The findings in other patients indicated that the great majority of the acute respiratory infections considered to be clinical influenza in this vicinity in 1949–50 were probably not infections with the usual influenza A or B viruses.

human beings: differentiation of specific and nonspecific type reactions. Australian J. Exper. Biol. & M. Sc. **24**: 207–211.

22. Dudgeon, J. A., Stuart-Harris, C. H., Glover, R. E., Andrewes, C. H. and Bradley, W. H. 1946, Influenza B in 1945–46. Lancet, **2**: 623–631.
23. Finland, M., Barnes, M. W., Meads, M., Ory, E. M. and Rubenstein, A. D. 1947, Influenza B in Needham, Massachusetts, December, 1945. Serologic studies in cases and in family contracts. J. Lab. & Clin. Med. **32**: 367–378.

THE FATAL ENTERIC CHOLERA INFECTION IN THE GUINEA PIG, ACHIEVED BY INHIBITION OF NORMAL ENTERIC FLORA

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The mechanism by which *Vibrio cholerae*, as well as other enteric pathogens, produce the symptom complex of diarrhea is poorly understood. Burrows, Wagner and Mather (1944) demonstrated that vibrio endotoxin increases the diffusion of fluid through living semipermeable membranes. Later, Burnet and Stone (1947) found that vibrio mucinase desquamates guinea pig intestine in vitro, and the possible role of this enzyme in the pathogenicity of cholera has also been discussed (Jensen, 1953; Raffel, 1953). Intensive studies have been done by others on the pharmacological action of bacterial endotoxins (literature summarized lately by Burrows, 1951). All these workers, however, used the parenteral injection of endotoxin preparation into animals. Their results are for this reason only partly applicable to conditions in the actual diseases, especially cholera, where endotoxin is liberated mainly into the lumen of the bowel. The relative severity and the time of onset of the different symptoms observed after parenteral doses of endotoxin will presumably be different when the endotoxin is resorbed from the intestine. It has, therefore, not been established yet whether the symptoms observed in human cholera are due to direct or central action of endotoxin (De, Sarkar and Tribedi, 1951) or whether they are caused merely by the extreme dehydration from diarrhea. The latter interpretation is strongly supported by the good therapeutic effects of salt solutions.

These and related problems have remained largely unsolved because of the lack of an experimental animal in which an enteric infection, simulating the human disease, could be regularly produced. *V. cholerae* is the only organism for which successful enteric animal infections have been reported: Nicati and Rietsch (1885) produced a fatal disease in rabbits by injecting live vibrios directly into the duodenum. Later, Koch (1885) pointed out that the fatality of this experimental disease depended on the amount of trauma inflicted by the operation. Koch reported successful enteric infection of guinea pigs by feeding vibrio cultures together with sodium carbonate and simultaneous inhibition of intestinal motility with opium tincture. Burrows, Elliot and Havens (1947) however, were unable to obtain consistent results with this method, even though a symptomless infection of guinea pigs could be achieved regularly. Their results were confirmed in the present studies. Even massive doses (30 mg) of young, live cholera vibrios injected in buffered alkaline suspensions directly into different parts of guinea pig intestine failed to produce any signs of disease. Metchnikoff (1894) was able to infect suckling rabbits with cholera. Sanarelli (1921) confirmed these results but was unable to infect older rabbits. Cantacuzene and Marie (1914) reported successful infection after irritating the intestine of guinea pigs with podophyllin, given orally or intraperitoneally Their results could not be reproduced in this laboratory. The dosage of podophyllin given by these authors proved

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to be toxic, while lesser amounts did not result in infection. Nasta (1914) was able to produce enteric cholera infection in guinea pigs by irritating the bowel with antiserums to intestinal mucosa. His results could not be reproduced in this laboratory.

The study described here is concerned with the production of an enteric infection in the guinea pig following intragastric administration of *V. cholerae* that is made possible by the use of streptomycin for the elimination of normal intestinal flora (Freter, 1954a), and with the immunity demonstrable against this kind of infection.

A. THE METHOD OF INFECTION

The sudden change in susceptibility to enteric cholera infection of rabbits after weaning can be taken as an indication that either the kind of diet or the composition of the normal flora, which is presumably related to it, might be an important factor in determining the resistance of an animal. The inconsistency of results in Koch's method might be explained on the same basis.

Starting from this hypothesis, it was found that guinea pigs could be infected regularly after the normal flora had been reduced by starvation and inhibited by administration of streptomycin. The infecting agent was a streptomycin-resistant cholera vibrio. This strain (C-11) had been isolated in early 1953 in Calcutta from a nonfatal case of cholera. Streptomycin resistance had been induced by four passages on gradient plates of veal infusion agar with increasing amounts (50 μg/ml to 4 mg/ml) of steptomycin in the lower layer. The strain was originally sensitive to 50 μg/ml of streptomycin; the resistant variant tolerated concentrations to 200 mg/ml of streptomycin.

After a number of preliminary experiments, the following procedure for infection has been adopted and used in all experiments described hereafter. It consists of four steps:

(1) The animals were starved for 4 days but water was given ad libidum throughout the experiment. It may be emphasized, in this connection, that guinea pigs are little affected by starvation. Animals of 350 to 500 g in weight, which were used in these experiments, did not show any signs of weakness after being without food for as long as 8 days.

(2) The second step in the procedure was the administration of 250 mg $CaCO_3$ suspended in 10 ml of distilled water by stomach tube.

(3) Three hours later the streptomycin-resistant vibrios were given, also by stomach tube, in 15 ml of veal infusion broth (previously diluted with distilled water to half strength), together with 250 mg $NaHCO_3$ and 5 mg streptomycin sulfate. The vibrios were taken from 16-hour-old veal infusion agar (containing 2 mg/ml streptomycin) cultures grown at 37 C. Bacterial counts of the inoculum were made by dilution in veal infusion agar, containing 2 mg/ml streptomycin.

(4) One-half hour later, 8 mg morphine sulfate were given intraperitoneally to reduce intestinal motility.

Some animals showed signs of disease by 12 hours after infection. With small infecting doses, the onset of symptoms may be delayed up to 48 hours. The animals became listless, and gradually paralysis of the hind legs, hypothermia and tremor developed. Death occurred within 18 to 48 hours. The cecum, and often the entire bowel, was found to be filled with 50 to 60 ml of fluid, containing *V. cholerae* in practically pure culture. This infection was strictly limited to the lumen of the bowel, and heart blood and spleen cultures were sterile. Control animals did

not show any free liquid in the bowel, the 25 ml given during the infection procedure being completely absorbed within a few hours. Normal diet was fed to surviving animals 36 hours after infection.

This infection resembled the human infection in three ways: (1) It was localized in the bowel; (2) it was frequently fatal; and (3) large amounts of fluid penetrated into the lumen of the bowel. The main difference between human and experimental infection lay in the necessity for inhibiting the normal flora and intestinal motility of the guinea pigs. In this respect it might be worthwhile to consider the possibility of inhibitory action on the part of the normal human enteric flora as a factor in the resistance of humans to enteric diseases. This theory has—to the knowledge of the author—first been discussed by Nissle (1916). Following this line of thought, the virulence of an enteric pathogen would depend to an appreciable degree upon its ability to overgrow the normal flora of the host. Consequently, the experimental guinea pig infection with *V. cholerae* would resemble human cholera from the moment when the vibrios have established themselves in the intestine. Future investigations using experimental guinea pig infections with mixed cultures might bring more light to this problem.

The action of morphine in promoting the experimental guinea pig infection has been assumed to be that of reducing intestinal motility; however, this has not been investigated experimentally so far and other possible mechanisms, like reduction of intestinal secretions, cannot be excluded. Two pen-bred strains of guinea pigs from a local dealer have been tested for susceptibility to cholera infection. In a simultaneous experiment, involving 15 guinea pigs of each strain, the LD_{50} was found to be about 1000 million vibrios for one strain and approximately 100,000 vibrios for the other. This difference in susceptibility might be due to a different composition of normal flora in the two strains. It seems more likely, however, that antagonism of the normal flora, which is largely eliminated in this method of infection, is not the only factor responsible for the resistance of laboratory animals to enteric infection. This question has not been further investigated.

B. PROTECTION OF ANIMALS BY IMMUNIZATION

The respective roles of endotoxin and mucinase in experimental cholera have been investigated by challenging guinea pigs which had been actively immunized with these agents. The experiments were conducted as follows:

Groups of 60 to 80 guinea pigs which had been received in one shipment, and were presumably of comparable susceptibility, were divided into two groups of equal individual weight, i.e., for each animal of weight X in one group there was an animal of the same weight in the other group (except in experiment #2, table 1). One group served as normal controls, while the other animals were immunized by the intraperitoneal inoculation of boiled vibrios or vibrio mucinase preparations at 5-day intervals. The number of injections given is indicated for each experiment in table 1. The preparations used for immunization were: *Boiled vibrios.*—The same streptomycin-resistant strain as used for infection was grown on veal infusion agar containing 2 mg/ml streptomycin for 18 hours at 37 C. The vibrios were suspended in saline and steamed without pressure for 2 hours. The suspension was diluted to contain 6 mg/ml dry weight of vibrios. One-half ml of this suspension was given in each injection. *Mucinase.*—The streptomycin-resistant

TABLE 1.—*Infection of immunized animals.*

Exp. no.	Weight of animals after starvation (g)	Immunized against	Number of antigen injections	Number of vibrios given on infection, and death of animals‖						LD_{50} Reed and Muench (1938)
1	270–380*	Normal controls	0	4.62×10^9	231×10^8 1/3	11.5×10^8 0/4	577×10^6 0/3	26×10^6 0/4	1440 0/4	
		Boiled vibrios	6	4/6	0/5	1/6	0/6	0/5		1.55×10^9
2	230–330†	Normal controls	0	36×10^9 6/6	1.8×10^9 5/7	90×10^6 6/7	4.5×10^6 3/6	225×10^3 4/7	11,250 2/7	1.0×10^6
		Boiled vibrios	4	3/5	2/4	2/6	1/5	2/4	1/4	221×10^6
3	270–400‡	Normal controls	0	339×10^6 2/4	16.8×10^6 8/8	840×10^3 4/4	42×10^3 3/4	2100 1/5		26×10^3
		Mucinase	6	3/5	3/6	2/5	0/7	0/5		16.8×10^6
4	240–330§	Normal controls	0	7.2×10^9 6/6	360×10^6 5/7	18×10^3 5/6	900×10^3 4/7	45×10^3 2/6	2250 2/6	426×10
		Mucinase	4	4/4	3/5	3/5	3/6	0/6	0/6	6.6×10

* It was noted that the animals in the normal control group had gained weight much faster than the immunized animals. At the time of infection, a number of controls had to be discarded because their weight was no longer comparable to that of the immunized group.

† For the above reason (*), the animals in the immunized group had been selected such that their average weight was 30g higher than that of the normal group. At the time of infection the average weight of the control group was slightly higher than that of the immunized group.

‡ Animals immunized with mucinase gained weight as fast as the normal guinea pigs. The immunized and control groups were, therefore, of equal weight at all times.

§ 4/6.—Four animals out of a group of six died of enteric cholera infection.

strain was grown on thionin glycerol agar (Husain, 1955) for 16 hrs. The agar was then mixed with distilled water, emulsified in a blendor and centrifuged to give a clear supernate of high mucinase activity which was lyophilized. The mucinase titer was determined with ovomucin as described elsewhere (Jensen, 1953; Freter, 1955a). Increasing amounts (0.5 to 2 ml) of this preparation, reconstituted with distilled water containing 0.01% merthiolate to a mucinase titer of 1:128, were injected for immunization.

The immunized animals were starved 3 days after the last injection and infected 4 days later (7 days after the last injection). The control group of each experiment was starved and infected concurrently. The results of four experiments are given in table 1.

As can be seen from experiment #1, table 1, there is no detectable protection in the group immunized with boiled vibrios. This result might, however, be due to the fact that overwhelming doses had to be given to the relatively unsusceptible animals, thus masking any immunity that might have been present. Experiment #2, was therefore, as all following experiments, carried out on the more susceptible breed of guinea pigs, mentioned above. The data of experiment #2 show a roughly 200-fold protection of the immunized group. Similar protection was found for the animals immunized with mucinase in experiments #3 and #4. The protection in experiment #4 is not as good as in experiment #3, a fact which might be

TABLE 2.—*Agglutinin titers of serums from immunized (boiled vibrios) and normal control animals.*

	Serums from immunized guinea pigs						Serums from normal guinea pigs					Antigen control with anticholera O rabbit serum
Animal no.	1	2	3	4	5	6	7	8	9	10	11	
Agglutinin titer	1:160	1:80	1:80	1:40	1:80	1:80	No agglutination in dilution 1:20					1:1600

TABLE 3.—*Agglutinin titers and fractional mucinase inhibition of serums from immunized (mucinase) and normal guinea pigs.*

	Serums from immunized guinea pigs				Serums from normal guinea pigs			Antigen control with cholera anti O rabbit serum
Animal no.	1	2	3	4	5	6	7	
Agglutinin 1:40 titer	No agglutination in 1:20				No agglutination in 1:20			1:800
Fractional inhibition of mucinase	1/8	1/16	1/8	1/16	1	1	1	1

due to the fewer antigen injections (4 instead of 6) given.

Some surviving animals of normal and control groups in experiment #2, which on infection had received the two smallest amounts of vibrios (11,250 and 225,000), were bled from the heart 3 days after infection and the agglutinin titers of the serums were determined. Agglutinating antigen was the same as immunizing antigen (table 2).

A similar test was carried out in experiment #3. In this case the fractional inhibition of mucinase* by these sera was also tested. The mucinase was the same as used for immunization (table 3).

Table 3 shows that the agglutinating antibody which had developed in the animals during immunization with mucinase preparation (which is presumably contaminated with O antigen) is appreciably less than in the case of immunization with boiled vibrios (table 2). This is remarkable because the protection against infection was at least equal in both cases. Table 3 indicates further that the serums of all immunized animals had an appreciable anti-mucinase activity. It can be considered as established (Jensen, 1953) that injection of boiled vibrios does not give rise to mucinase antibodies in the serums of immunized animals. For this reason, the mucinase titrations had been omitted in experiment #2 (tables 1 and 2).

In general, then, immunization with boiled vibrios or mucinase preparation gave demonstrable protection. In the case of mucinase immunization, it cannot be decided whether this protection is due to contamination of the mucinase used for immunization with other vibrio antigens or with other enzymes. The low agglutinin titer in protected animals which had been immunized with mucinase (table 3) as compared to the agglutinin titer of protected animals immunized with boiled vibrios (table 2) suggests, however, that some factor other than agglutinating O-antigen in the mucinase preparations might give effective protection. Conclusive results can apparently be obtained only by passive immunization experiments with absorbed serums.

C. TESTS ON FLUID COLLECTED FROM THE INTESTINES OF INFECTED ANIMALS

The significance of death of animals which had been infected with only small amounts of vibrios has been investigated by making bacterial counts of the liquid collected from their in-

* $\text{Fractional inhibition} = \frac{\text{mucinase titer in normal serum}}{\text{mucinase titer in the tested serum}}$.

Example: The mucinase titer in 5% normal rabbit serum was 1:512. In 5% serum from animal #1, it was 1:64. The value of fractional inhibition given in table 3 for this serum is therefore

$$\frac{1:512}{1:64} = \frac{64}{512} = \frac{1}{8}$$

meaning the tested serum depressed the mucinase activity to an eighth of its original value.

TABLE 4.—*Number of living vibrios recovered from fluid in the bowel of animals which died of enteric cholera infection.*

Animals immunised with mucinase:		*Normal control animals:*	
Number of vibrios given on infection	Number of vibrios recovered:	Number of vibrios given on infection:	Number of vibrios recovered:
336 $\times 10^6$	25 $\times 10^9$	336 $\times 10^6$	4.4 $\times 10^9$
336 $\times 10^6$	15 $\times 10^9$	336 $\times 10^6$	1.2 $\times 10^9$
336 $\times 10^6$	10 $\times 10^9$	16.8 $\times 10^6$	12 $\times 10^9$
16.8 $\times 10^6$	19.5 $\times 10^9$	16.8 $\times 10^6$	4 $\times 10^9$
16.8 $\times 10^6$	7.5 $\times 10^9$	840 $\times 10^6$	1.3 $\times 10^9$
16.8 $\times 10^6$	400 $\times 10^6$	42 $\times 10^6$	10 $\times 10^9$
16.8 $\times 10^6$	300 $\times 10^6$	42 $\times 10^6$	45 $\times 10^6$
840 $\times 10^6$	100 $\times 10^6$	42 $\times 10^6$	250 $\times 10^6$
840 $\times 10^6$	80 $\times 10^6$	2100	26 $\times 10^6$

testines. The counts were made in veal infusion agar containing 2 mg/ml streptomycin. The number of vibrios recovered has been calculated for the amount of liquid in the intestines. Table 4 gives the results of one determination of this kind, carried out on animals of experiment #3, described above (table 1).

The data in table 4 indicate a considerable multiplication of vibrios in the intestine, especially in the case of small inoculums. No difference was found between immunized and normal animals, suggesting that once the disease is acquired, its severity is not influenced by immunization, even though there was protection against infection (table 1, experiment #3). It may be noted in this connection that only animals which, after death, had considerable amounts (over 30 ml) of fluid in their intestines have been considered in the tables of this paper. A few animals which died without this symptom have been disregarded.

The liquid collected from the intestines of infected animals of experiment #3, table 1, was also tested for mucinase activity. The mucinase titers ranged from 1:64 to 1:1024 and were found to be of the same order in fluids from both normal and immunized animals. This shows again, that immunization, though protective against infection, did not alter the course of the disease once it had been acquired. This conclusion is further supported by the fact that the time of death after infection was not different for normal animals or animals immunized with either mucinase or boiled vibrios.

The mucinase activity in the intestinal fluid of infected animals was specifically inhibited by antiserums prepared in rabbits with vibrio mucinase, while normal rabbit serum had none or little effect. This shows that the mucinase activity is—at least to an appreciable degree—of vibrio origin.

D. THE EFFECT OF ORAL DOSES OF ENDOTOXIN

As mentioned above, the question of whether or not endotoxin is responsible for the symptoms and death in cholera is still a matter of considerable speculation and little experimental evidence is available. Buergers (1910) was unable to kill guinea pigs by feeding very large amounts of heat-killed cholera vibrios. This result has been confirmed in this laboratory. It was also found here that mice were resistant to large oral doses of endotoxin in the form of "acid soluble toxin" (Freter, 1955b) or heat-killed vibrios. The natural resistance of these experimental animals to cholera infection prohibits, however, any inference as to what may happen in man under similar conditions. This objection might be overcome with the present method of making guinea pigs susceptible to enteric cholera infection.

Two experiments have, therefore, been carried out in which guinea pigs underwent the same procedures required for enteric cholera infection, with the exception that heat killed vibrios or endotoxin preparations were given instead of living vibrios.

(1) In the first experiment, 285 mg

TABLE 5.—*The effect of oral doses of endotoxin.*

Amount of endotoxin given *orally* (in mg dry weight of vibrios)	1200	600	300	0	0	0
Amount of endotoxin given *intraperitoneally* (in mg dry weight of vibrios)	0	0	0	190	80	33
Deaths	0/5	0/4	0/4	4/4	4/4	4/4
Symptoms:	Animals perfectly normal, sacrificed 48 hours after injection. No fluid in intestines			Marked hypothermia, paralysis of hind legs and tremor. No fluid in intestines		

of heat-killed vibrios were fed to four guinea pigs. Two control animals which also had undergone the complete infection procedure, but had received no vibrios at all orally, were given 60 mg of the same heat-killed vibrios intraperitoneally. The latter two animals died overnight. On autopsy, no liquid was found in their intestines. The four animals which had received oral doses of heat-killed vibrios were completely normal. One of these guinea pigs was sacrificed 24 hours after injection. No liquid was found in the intestines. The other three animals were killed 48 hours after injection and also had no liquid in their intestines.

It may be emphasized, in this connection, that the dose of 285 mg dry weight of vibrios given orally in this experiment contains about as many vibrios as can be expected to grow in a guinea pig of 300 to 400 g weight during the actual infection. One might object that the endotoxin in this experiment was contained within the vibrios and did not diffuse into the bowel, while in the case of intraperitoneal injection the environmental conditions at that site favored such a diffusion. The experiment was therefore repeated, using as endotoxin preparation a mixture of "acid soluble toxin" (Freter, 1955b) plus heat-killed vibrios.

(2) The vibrios (same streptomycin-resistant strain as used for infections) were grown in aerated peptone water and collected in a Sharples centrifuge as described elsewhere. They were resuspended in distilled water to 95 mg/ml dry weight, adjusted with HCl to pH 3.8, incubated for 4 hours at 37C, readjusted with NaOH to pH 7 and steamed without pressure for 30 minutes.

The toxicity of this suspension was first tested on intraperitoneal injection in mice. The LD_{50} was found to be 0.0625 ml, corresponding to 5.94 mg dry weight of vibrios. A sample of this suspension was centrifuged and the supernate tested similarly in mice. The LD_{50} was less than 0.0625 ml, indicating that at least most of the toxicity was in solution. The suspension proved to be sterile in tests on veal infusion agar plates.

The suspension was then administered orally or intraperitoneally to guinea pigs which had undergone the complete infection procedure, except that living vibrios had been omitted. The results are given in table 5. These results indicated that large doses of soluble endotoxin had no effect on animals which were susceptible to enteric cholera infection. The toxicity for mice of the vibrio suspension used was admittedly rather low (LD_{50} = 5.94 mg). Nevertheless, the largest oral amount (1200 mg) given to guinea pigs was 36 times the smallest dose which killed four out of four animals when given intraperitoneally. This dose of 1200 mg corresponded to 300 mg of a highly toxic vibrio (LD_{50} for mice of around 1.5 mg dry weight) and contained, therefore, the maximal amount of endotoxin which could reasonably be expected to develop during the actual infection of a guinea pig.

The interpretation of these findings is rather difficult and several possibilities should be discussed:

(1) The number of vibrios which develop during the actual infection of a guinea pig is much higher than assumed here. However, the bacterial counts on intestinal fluids of infected animals (table 4) do not favor such an argument.

(2) The vibrios liberate another form of endotoxin during actual infection which unlike the toxin extracted in vitro at low acidity is resorbed from the guinea pig intestine. The in vitro extraction of two kinds of endotoxin ("acid soluble" and "acid insoluble"), described elsewhere (Freter, 1955b), favors this hypothesis. Furthermore, the symptoms exhibited by animals which had received intraperitoneal injections of endotoxin (hypothermia, paralysis of hind legs, tremor) are strikingly similar to those encountered with infected animals, which suggests a significant role of endotoxin in the experimental disease. The accumulation of fluid in the intestine might then, in accordance with this hypothesis, be explained as a side effect of the postulated form of endotoxin or as a result of action of another unrelated agent, produced by the vibrios.

(3) The vibrios produce another agent (mucinase?) which alters the permeability of the bowel and thus permits the endotoxin to enter the circulation. The same agent might be responsible for the diffusion of fluid into the lumen of the intestine. This third hypothesis is favored by the constant occurrence of high titer mucinase in the intestinal fluids of infected animals.

No decision in favor of any one of these hypotheses can as yet be made. However, further similar experiments using passive immunization or administration of enzyme preparations might well furnish sufficient evidence to definitely describe the actual mechanism of the pathogenesis of the cholera vibrio.

SUMMARY

Starting from the hypothesis that antagonism of the normal bacterial flora might be a significant factor in the natural resistance of laboratory animals to enteric infections, a method of infecting guinea pigs with cholera has been developed in which the normal flora of the animals is inhibited by streptomycin. Infecting agent is a streptomycin-resistant vibrio strain. This experimental infection is fatal and limited to the bowel. Accumulation of fluid occurs in the intestines of infected animals.

Two breeds of guinea pigs have been tested for their susceptibility. The LD_{50} was around 100,000 vibrios for one breed while the other breed was much more resistant (LD_{50} around 1 billion vibrios). This was taken as an indication that other mechanisms, beside bacterial antagonism, also play a significant part in determining the resistance of an animal to enteric cholera infection.

It was found that immunization with both boiled vibrios or mucinase preparations gave effective protection against infection. Whether the protection by mucinase immunization was actually due to the anti-enzyme or to antibodies to other antigens present in the mucinase preparations has not been determined.

Bacterial counts and mucinase determinations on the intestinal fluids of infected animals indicate that both types of immunization—though protective—did not alter the course of the disease once it had been acquired. This conclusion is further supported by the fact that the time of death after infection was not different for normal or immunized animals.

Large doses of endotoxin in the form

of heat-killed vibrios or of water soluble toxin preparation did not affect guinea pigs when given orally. A number of explanations for these findings are discussed.

REFERENCES

Buergers, T. 1910, Ueber das Choleragift. Verhandl. d. Ges. Dt. Naturf. u. Aertze. **82**: 521–524.

Burnet, F. M. and Stone, J. D. 1947, Desquamation of the intestinal epithelium in vitro by *Vibrio cholerae* filtrates. Australian J. Exper. Biol. **27**: 245–252.

Burrows, W. 1951, Endotoxins. Ann. Rev. Microbiol. **5**: 181–196.

Burrows, W., Elliot, M. and Havens, I. 1947, Studies on immunity to Asiatic cholera. IV. J. Infect. Dis. **81**: 261–281.

Burrows, W., Wagner, S. M. and Mather, A. N. 1944, The endotoxin of the cholera vibrio: Action on living semipermeable membranes. Proc. Soc. Exper. Biol. & Med. **57**: 311–314.

Cantacuzene, J. and Marie, A. 1914, Cholera gastrointestinal experimental chez la cobaye. Comp. rend. Soc. de biol. **76**: 307–310.

De, S. N., Sarkar, J. K., and Tribedi, B. P. 1951, An experimental study of the action of cholera toxin. J. Path. & Bact. **63**: 707–717.

Freter, R. 1954a, Fatal enteric cholera infection in the guinea pig. Bact. Proc. 1954, p. 56.

———. 1955a, The serologic character of vibrio mucinase. J. Infect. Dis. In press.

———. 1955b. In preparation.

Husain, S., 1955, Thesis, University of Chicago.

Jensen, K. E. 1953, Immunologic characterization of a mucolytic enzyme of *Vibrio cholerae*. J. Infect. Dis. **93**: 107–110.

Koch, R. 1885, Zweite Konferenz zur Eroerterung der Cholerafrage. Deutsche med. Wchnschr. **11**, no. 37A, pp. 1–60.

Metchnikoff, E. 1894, Recherches sur le cholera et les vibrions. Ann. Inst. Pasteur, **8**: 529–589.

Nasta, M. 1914, Cholera experimental chez des cobayes ayant recu prealablement une injection de serum enterolytique. Compt. rend. Soc. de biol. **77**: 177–178.

Nikati and Rietsch, 1885, Recherches sur le cholera. Experiences d'inoculation Rev. Med. **5**: 449–462.

Nissle, A. 1916, Ueber die Grundlagen einer ursaechlichen Bekaempfung der pathologischen Darmflora. Deutsche med. Wchnschr. **42**: 1181–1184.

Raffel, S. 1953, Immunity, Hypersensitivity, Serology. New York, Appleton-Century-Inc., pp. 304–310.

Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**: 493–497.

Sanarelli, G. 1921, De la pathogenie du cholera V. Le "cholera intestinal" des jeunes animaux. Ann. Inst. Pasteur, **35**: 745–796.

THE POSITION OF *CLOSTRIDIUM TETANI* WITHIN THE SEROLOGICAL SCHEMA FOR THE PROTEOLYTIC CLOSTRIDIA

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In a serological study of strains of *Clostridium sporogenes*, Mandia and Bruner[1] showed that identification of proteolytic clostridia was possible by means of antigenic formulas based on the reactions of the heat-labile antigens. Of the 25 *Cl. sporogenes* strains which they studied, 18 formed a homogenous serological group and were referred to as group I of the proteolytic clostridia. Three strains designated as *Cl. sporogenes* formed a second group with a strain of *Clostridium parabotulinum*. When a number of strains of *Cl. parabotulinum* and related organisms were included in the study, all reacted as members of the second group[2] This group was then referred to as serological group II of the proteolytic clostridia. A strain designated as *Cl. sporogenes* did not fall into either groups I or II, but showed, however, an agglutinative relationship to a strain of *Clostridium histolyticum*. When 13 strains of *Cl. histolyticum* were included in the study, they demonstrated none of the 12 previously determined antigenic factors possessed by organisms of groups I and II and they comprised a third homogenous serological group of the proteolytic clostridia, group III[3]

Serological identification of representatives of these three species was based upon the production of a floccular H type agglutination. The proteolytic clostridia, however, apparently demonstrate three types of agglutination[4]: (1) a floccular (H type) agglutination occurring with motile strains after 1 hour incubation at 50 C; (2) a coarsely granular heat-labile somatic type of agglutination occurring with motile and nonmotile strains after 3 hours incubation at 50 C (with motile strains this type of agglutination remains after the H floccules have been resuspended); (3) a finely granular heat-stable somatic type of agglutination occurring after 24 hours incubation at room temperature.

The heat-labile somatic antigen of the proteolytic clostridia may be spoken of as the L antigen and the heat-stable antigen as the O antigen in accordance with the terminology of Henderson.[5] He found the two somatic antigens to be serologically distinct in the nonmotile species *Clostridium welchii*, the L antigen producing a coarsely granular and the O antigen a finely granular agglutination, both types appearing after 4 hours of incubation at 43 to 45 C. It appears that the L and O antigens of the proteolytic clostridia are species-specific, while the H antigen is type-specific. Four strains identified as *Cl. parabotulinum* were studied, however, which possessed the L and H antigens (assigned factors 5 and 6, respectively) of *Cl. parabotulinum* and the O antigen (assigned the group Roman numeral factor III) of *Cl. histolyticum*.[4] The significance of this antigenic crossing be-

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1. Mandia, J. W. and Bruner, D. W. 1951, J. Immunol. **66**: 497–505.
2. Mandia, J. W. 1951. J. Immunol. **67**: 49–55.
3. Mandia, J. W. 1952, J. Infect. Dis. **90**: 48–50.
4. Mandia, J. W. 1952, J. Immunol. **69**: 451–455.
5. Henderson, D. W. 1940, J. Hyg. **40**: 501–512.

tween two distinct species of proteolytic clostridia at present is not understood.

In the serological identification of proteolytic clostridia those organisms considered strains of *Cl. sporogenes* which do not react in the antiserums to any one of the groups I (*Cl. sporogenes*), II (*Cl. parabotulinum*) or III (*Cl. histolyticum*) serotypes are compared serologically with typical representatives of other proteolytic clostridial species, such as *Clostridium bifermentans*, *Clostridium sordelli*, *Clostridium tyrosinogenes* and *Clostridium tetani*. A nontoxic organism isolated by Dr. Louis D. Smith and identified as *Cl. sporogenes* S-17 was studied, which was not related antigenically to any of groups I, II or III serotypes, but demonstrated, however, an agglutinative relationship to a strain of *Cl. tetani*.

It was felt that perhaps other strains of the species *Cl. tetani* might demonstrate antigenic relationship to the *Cl. sporogenes* strain S-17 and form a homogenous group within the serological schema for proteolytic clostridia in a manner similar to that previously described for representatives of the species *Cl. sporogenes*, *Cl. parabotulinum* and *Cl. histolyticum*.

Apparently an agglutinative relationship between organisms considered *Cl. sporogenes* and the species *Cl. tetani* has not been observed or reported previously in literature. According to Gunnison[6] and MacLennan,[7] the eight recognized H types and the nonmotile type VI of *Cl. tetani* possess an O antigenic relationship to several other species, notably *Clostridium tetanomorphum*; however, the *Cl. tetani* types possess a species specific O factor demonstrable by agglutinin absorption.

The purpose of this investigation was to study the antigenic structure of proteolytic strain S-17 designated *Cl. sporogenes*, and strains of *Cl. tetani* and, if possible, to determine their position within the serological schema for proteolytic clostridia.

MATERIALS AND METHODS

According to Smith,[8] strain S-17 (*Cl. sporogenes*) was isolated in the following manner: Tubes of chopped meat with 1% glucose were inoculated with small samples (0.5 to 1 g) of soil, heated in a boiling water bath for a few minutes, and incubated overnight. About 0.5 ml of supernatant fluids was injected intramuscularly into guinea pigs, which died within 24 to 48 hours. Blood plates were streaked from infected muscle at some distance from the site of injection incubated anaerobically for 2 days, and medusa head colonies typical of the species *Cl. sporogenes* were selected for study. Cultures from these colonies were actively proteolytic, motile, fermented glucose and maltose and were nontoxic. The purpose of the experiment was to isolate strains of *Cl. sporogenes* which invaded muscular tissue.

Sixteen strains of *Cl. tetani* were studied. These strains were obtained through the courtesy of Miss Janet Gunnison. The strains were labelled according to type. Strain 1 was labelled type I; strain 2, type II; strains 3, 4 and 5, type III; strain 6, type IV; strains 7, 8 and 9, type V; strain 10, type VI; strain 11, type VII; strains 12, 13 and 14, type VIII; strains 15 and 16, type IX. Strains of *Cl. sporogenes* 291 (group I), *Cl. parabotulinum* 455 (group II) and *Cl. histolyticum* H-13 (group III), representative of each of the three serological groups of the proteolytic clostridia, were included in the study. The sixteen strains of *Cl. tetani* were cultured in brain liver heart semisolid medium for 48 hours and plated on liver veal agar. A layer of agar approximately 2 mm thick was poured over each streaked plate in order to eliminate spreading and to obtain discrete colony formation. The subsurface colonies formed after 48 hours incubation in a Brewer hydrogen jar were woolly in appearance. Single colonies of each strain were picked into brain liver heart semisolid medium and incubated 48 hours and replated. After three successive platings and colony selection, the final organism obtained for each strain was the stock culture employed in these studies.

The 16 *Cl. tetani* strains were found to possess

6. Gunnison, J. B. 1937, J. Immunol. **32**: 63–74.
7. MacLennan, J. D. 1939, Brit. J. Exper. Path. **20**: 371–376.
8. Smith, L. D. 1953, Personal communications.

the typical biochemical, cultural and toxigenic characteristics described for the species. That is, they were motile except for strain 10, which was nonmotile type VI; fermented no sugars; produced indol and H_2S; liquefied gelatin, and upon intramuscular injection of guinea pigs, they produced tetanospasms followed by death within 48 hours. The proteolytic activity of the *Cl. tetani* strains was investigated in coagulated egg-albumen, blood serum, milk and egg-meat medium. Slight growth occurred but evidence of proteolysis was absent in tubes incubated without the use of special apparatus. Heavier growth and proteolysis was apparent in tubes incubated in a Brewer hydrogen jar after 15 to 30 days. In an attempt to obtain evidence of proteolysis of strains of *Cl. tetani* cultivated without the use of special apparatus, powdered reduced iron was added to the protein mediums. Hastings and McCoy[9] found that added powdered reduced iron greatly improved milk as a medium for cultivation of anaerobes. The proteolytic activity of the *Cl. tetani* strains in these mediums with iron incubated aerobically, was greater than that shown in the same medium without iron in a Brewer jar. Apparent digestion in each medium became visible after 7 to 10 days incubation at 37 C. The proteolytic acitvity of *Cl. sporogenes* 291, *Cl. parabotulinum* 455, *Cl. histolyticum* H-13 and *Cl. sporogenes* S-17 in the iron-protein medium was greater than that of *Cl. tetani*. The former species produced almost complete digestion of the iron-protein mediums after 7 to 10 days incubation.

In order to obtain cellular suspensions of the *Cl. tetani* strains and *Cl. sporogenes* S-17 with a maximum development of the flagellar H antigen, a semisolid medium was inoculated with each strain; incubated for 48 hours, and 1 ml of the actively motile organism at the base of the tubes was pipetted into flasks containing 400 ml of horse meat infusion broth.[1] After 24 hours incubation the broth cultures were formolized to a concentration of 0.4%. These formolized whole broth cultures of the 16 *Cl. tetani* strains were the heat-labile antigens used in the agglutination tests and for the preparation of antiserums. Similar broth cultures heat-killed at 100 C for 2 hours and formolized as employed in previous studies.[1,4] produced autoagglutinable heat-stable antigenic suspensions of the *Cl. tetani* strains. It was found that by separating the heated *Cl. tetani* cells from the broth culture by centrifugation and resuspending in 100 ml of 0.4% formolized saline, stable heated cellular suspensions were obtained.

This method of preparing the heat-stable antigens of the *Cl. tetani* strains was used also in the preparation of the heat-stable antigen of *Cl. sporogenes* S-17. The heat-stable antigenic suspensions were used undiluted in the preparation of antiserums and diluted 1:4 with saline in the agglutination tests. Antiserums were prepared to the heat-labile and heat-stable antigens of each of the 16 *Cl. tetani* strains and *Cl. sporogenes* S-17 by injection of rabbits according to the method previously described.[1] All cellular suspensions used in the preparation of antigens and antiserums were first checked microscopically for the degree of sporulation. In all preparations the presence of spores or sporeforms was rare or entirely absent. The serological behavior of these antigenic suspensions, therefore, was considered due to that of the vegetative cells.

RESULTS

In the study of the heat-labile antigens, the formolized whole broth cultures were tested at dilutions of 1:20 to 1:5000 of antiserums. After 1 hour incubation at 50 C the tubes were read for H agglutination and then they were incubated further for 2 hours and read again for L agglutination. In tubes containing both H and L types, the L agglutination was distinguished from H agglutination by gentle shaking which resuspended the H floccules and left the coarsely granular L clumps intact.

In general, the specific heat-labile component in the 8 motile types of *Cl. tetani* produced high titers (1:1000 to 1:2000) and floccular H agglutination, while a group component produced low titers (1:20 to 1:500) and coarsely granular L agglutination (table 1). Strains representing types II, III, IV and V formed one group, while strains representing types I, VI, VII, VIII and IX formed another group. Strains 1, 2, 3, 6, 8, 10, 11, 12 and 15 were selected to represent the *Cl. tetani* types I through IX, respectively. Several discrepancies occurred, however, between the type designations as received of strains 5, 7 and 14 and their type reactions obtained in this study. Strain 5, labelled type III when received, reacted as type V; strain

9. Hastings, E. G. and McCoy, E. 1932, J. Bact. **23**: 54–55.

TABLE 1.—*Reactions of the heat-labile antigens of strains of* Cl. tetani.

Antigens		Antiserums								
Strains		2	3	6	8	1	10	11	12	15
	Type	II	III	IV	V	I	VI	VII	VIII	IX
2	II	2000	100	60	100	—	—	—	—	—
3, 4, 7	III	100	2000	100	100	—	—	—	—	—
6	IV	50	50	5000	50	—	—	—	—	—
5, 8, 9, 14	V	50	100	100	2000	—	—	—	—	—
1	I	—	—	—	—	2000	200	500	500	100
10	VI	—	—	—	—	200	500	100	100	100
11	VII	—	—	—	—	1000	500	2000	50	20
12, 13	VIII	—	—	—	—	200	200	100	2000	500
15, 16	IX	—	—	—	—	50	200	100	1000	2000

—, negative dilution, 1:20.

7, labelled type V, reacted as type III. Strain 14, labelled type VIII, reacted as type V. These discrepancies in type designations were considered due to mislabelling of cultures and were disregarded.

The absorption of the antiserums to strains representing types II, IV and V by cells of strain 3, type III, and the absorption of the antiserum to strain 3, type III, by cells of strains representing types II, IV and V removed a group factor assigned numeral 15 (next in sequence in the serological schema) and left specific factors assigned numerals 16, 17, 18, and 19 for types II, III, IV, and V, respectively (table 2). Similarly, the absorption of the antiserums to types VI, VII, VIII and IX by cells of type I and the absorption of the type I antiserum by cells of each of the types VI, VII, VIII and IX removed a group factor assigned numeral 20; left specific factors 21 and 22 for types I and VII, respectively, and left a subgroup factor 23 common to types VIII and IX. Reciprocal absorption showed that types VIII and IX possessed specific factors assigned 24 and 25, respectively. After absorption there was no specific factor left in the antiserum to the nonmotile type VI.

The heat-labile antigenic factors were determined for *Cl. sporogenes* S-17, as shown in table 3. The heat-labile component of *Cl. sporogenes* S-17 was related to that found in the group made up of *Cl. tetani* types II, III, IV and V. The absorption of the antiserum to *Cl. sporogenes* S-17 by cells of *Cl. tetani* (strain 3) type III removed the heat-labile *Cl. tetani* group factor 15 and left a specific factor for S-17 assigned 26. The absorption of the antiserum to *Cl.*

TABLE 2.—*The determination of heat-labile antigenic factors for strains of* Cl. tetani.

Antiserums		Antigens								
Strains		2	3	6	8	1	10	11	12	15
	Type	II	III	IV	V	I	VI	VII	VIII	IX
	Factors	15, 16	15, 17	15, 18	15, 91	20, 21	20	20, 22	20, 23, 24	20, 23, 25
Strain 2 absorbed by 3	16	2000	—	—	—	—	—	—	—	—
3 absorbed by 2	17	—	1000	—	—	—	—	—	—	—
6 absorbed by 3	18	—	—	2000	—	—	—	—	—	—
8 absorbed by 3	19	—	—	—	2000	—	—	—	—	—
10 absorbed by 1	—	—	—	—	—	—	—	—	—	—
1 absorbed by 11	21	—	—	—	—	2000	—	—	—	—
11 absorbed by 1	22	—	—	—	—	—	—	1000	—	—
12 absorbed by 1	23, 24	—	—	—	—	—	—	—	1000	500
15 absorbed by 1	23, 25	—	—	—	—	—	—	—	500	1000
12 absorbed by 15	24	—	—	—	—	—	—	—	500	—
15 absorbed by 12	25	—	—	—	—	—	—	—	—	500

—, negative dilution, 1:20.

TABLE 3.—*Determination of the heat-labile antigenic factors for* Cl. sporogenes *strain S-17.*

Antiserums	Antigens					
		Cl. sporogenes S-17	*Cl. tetani* type III	*Cl. tetani* type IV	*Cl. tetani* type V	*Cl. tetani* type II
	Factors	15, 26	15, 18	15, 19	15, 20	15, 17
Cl. tetani type III	15, 18	500	2000	50	100	100
Cl. sporogenes S-17	15, 26	2000	200	100	100	100
S-17 absorbed by type III	26	2000	—	—	—	—
Type III absorbed by S-17	18	—	2000	—	—	—

—, negative dilution, 1:20.

tetani type III by cells of strain S-17 similarly removed the group factor 15 and left the specific factor 18 for *Cl. tetani* type III. *Cl. sporogenes* S-17, therefore, shares the group factor 15 of *Cl. tetani* and possesses a specific factor 26 for itself. Based upon this heat-labile antigenic relationship to *Cl. tetani*, *Cl. sporogenes* S-17 could be identified, perhaps, as a nontoxic form of a new type of *Cl. tetani*.

HEAT-STABLE ANTIGENS

Upon incubation at room temperature for 24 hours, the heat-stable antigens of *Cl. sporogenes* S-17 and the *Cl. tetani* types reacted in the antiserums used in a previous study[4] to *Cl. sporogenes* 291 (group I), *Cl. parabotulinum* 455 (group II) and *Cl. histolyticum* H-13 (group III) at titers below 1:100. The heat-stable antigens of *Cl. sporogenes* 291, *Cl. parabotulinum* 455 and *Cl. histolyticum* H-13, however, reacted in the antiserums to *Cl. sporogenes* S-17 and the 16 *Cl. tetani* strains at titers of 1:200 to 1:1000. It appeared that all of these strains possessed a heat-stable antigenic factor in common but this factor was only slightly represented in the agglutinin titers of the antiserums to strains 291, 455 and H-13 and better represented in the agglutinin titers of the antiserums to strain S-17 and the *Cl. tetani* strains. When antiserums were prepared for the heat-stable antigens of strains 291, 455 and H-13 in the same manner as for strain S-17 and the *Cl. tetani* strains, that is, from heat-stable antigens made from formolized saline suspensions of heated cells rather than from heated 24-hour broth cultures, they demonstrated a stronger common agglutinin factor giving titers above dilution 1:100. Accordingly, similar formolized saline suspensions of heated cells were prepared for 32 group I, 30 group II, and 13 group III strains and tested in these new antiserums.

All organisms studied representa-

TABLE 4.—*Determination of heat-stable antigenic factors for species of proteolytic clostridia.*

Antiserums	Antigens					
	Strain	*Cl. sporogenes* group I, 32 strains	*Cl. parabotulinum* group II, 30 strains	*Cl. histolyticum* group III, 13 strains	*Cl. tetani* 16 strains	*Cl. sporogenes* S-17
	Factors	I, VI	II, IV	III, IV	IV, V	IV, VI
Cl. sporogenes 291	I, IV	2000	500	500	500	1000
Cl. parabotulinum 455	II, IV	1000	2000	500	500	500
Cl. histolyticum H-13	III, IV	500	1000	2000	200	200
Cl. tetani III, strain 3	IV, V	500	500	200	5000	500
Cl. sporogenes S-17	IV, VI	1000	500	200	1000	2000
291 absorbed by 455	I	1000	—	—	—	—
455 absorbed by 291	II	—	1000	—	—	—
H-13 absorbed by 455	III	—	—	500	—	—
Cl. tetani 3 absorbed by 455	V	—	—	—	2000	—
S-17 absorbed by 455	VI	—	—	—	—	1000

—, negative dilution, 1:100.

tive of groups I, II and III of the proteolytic clostridia appeared to possess a common heat-stable factor as well as the specific heat-stable factors assigned Roman numerals I, II and III, as determined in a previous study.[4] Moreover, the *Cl. tetani* strains and *Cl. sporogenes* S-17 apparently shared this common factor. The titers of the groups I, II, and III specific factors (table 4) were higher (1:2000 to 1:5000) than that of their common factor (1:200 to 1:1000). The absorption of the antiserum to *Cl. sporogenes* 291 by heated cells of *Cl. parabotulinum* 455 removed the common factor assigned Roman numeral IV and left factor I specific for the group I strains. Similarly, the absorption of the antiserum to strain 455 by cells of strain 291 removed the common factor IV and left factor II specific for group II strains. The factor III specific for group III strains was demonstrated by the absorption of the antiserum to strain H-13 by heated cells of strain 455. The absorption of the antiserum to *Cl. tetani* strain 3 (type III) by heated cells of strain 455 removed the common factor IV and left a factor assigned V specific for the strains of *Cl. tetani*. The absorption of the antiserum to strain S-17 by heated cells of strain 455 removed the common factor IV and left a specific factor for strain S-17 assigned VI.

Apparently, in addition to species-specific antigenic factors, as determined in a previous study,[4] heated cells of representatives of groups I, II and III of the proteolytic clostridia also possess a common factor labelled IV. Sixteen strains of *Cl. tetani* and strain S-17 designated *Cl. sporogenes* possessed the factor IV and specific factors labelled V and VI. Based upon the reactions of the heat-stable antigens, it appears that the various species of proteolytic clostridia actually are very closely related. Moreover, strain S-17, like proteolytic clostridia which are recognized as distinct species, possesses the common factor IV and a specific factor (VI) for itself; accordingly, it should be considered as a representative of a species distinct from either *Cl. sporogenes*, *Cl. parabotulinum*, *Cl. histolyticum* or *Cl. tetani*.

FIG. 5.—*Positions of species serotypes of proteolytic clostridia.*

Serological group	Serotypes		Species
A	I, IV:1, 2 I, IV:1, 3 I, IV:1, 4		*Cl. sporogenes*
B	II, IV:5, 6 II, IV:5, 7, 8 II, IV:5, 7, 9 II, IV:5, 8, 10 II, IV:5, 11 II, IV:5, 12		*Cl. parabotulinum*
C	III, IV:13 III, IV:13, 14		*Cl. histolyticum*
D		Type	*Cl. tetani*
	IV, V:20, 21	I	
	IV, V:15, 16	II	
	IV, V:15, 17	III	
	IV, V:15, 18	IV	
	IV, V:15, 19	V	
	IV, V:20	VI	
	IV, V:20, 22	VII	
	IV, V:20, 23, 24	VIII	
	IV, V:20, 23, 25	IX	
E	IV, VI:15, 26 (Strain S-17)		
Organisms which antigenically indicate species crossing	III, IV: 5, 6		*Cl. parabotulinum* (infectious?)

It is apparent that the primary serological groupings of proteolytic clostridia should be based upon the reaction of the heat-stable antigen. Therefore, according to the system used in Salmonella typing and recommended in the International Code of Nomenclature, it is proposed to assign the alphabetic letters A, B and C to the three primary serological groups of proteolytic clostridia, replacing Roman numeral I, II and III, respectively, as used in earlier papers.[1–4] Also, it is proposed to include representatives of the species *Cl. tetani* within the serological schema as a new group D, and strain S-17 in another group, E, as a representative of a yet unrecognized species which is apparently

biochemically and culturally similar to *Cl. sporogenes* (group A) except that it can invade muscular tissue.

The proposed serological groups and their various species serotypes are shown in table 5. The 4 strains of the previously designated *Cl. parabotulinum* serotype III:5, 6 were found also to possess the common factor IV and the species factor III of *Cl. histolyticum*. For the present, these strains are placed in a group designated "organisms which antigenically indicate species crossing." It is possible that perhaps later they can be shown to be bacterial hybrids.

SUMMARY

Sixteen strains representative of the nine recognized types of *Clostridium tetani* shared a heat-stable antigenic factor IV common to the species *Clostridium sporogenes*, *Clostridium parabotulinum*, and *Clostridium histolyticum*. Upon absorption of the antiserums to representative strains of these four species, the agglutinin factor IV was removed and species specific factors labelled I, II, III and V remained for *Cl. sporogenes*, *Cl. parabotulinum*, *Cl. histolyticum*, and *Cl. tetani* strains, respectively.

An organism designated *Cl. sporogenes* S-17 shared the heat-labile antigenic factor 15 with the *Cl. tetani* types II, III, IV and V but not the heat-stable factor V specific for *Cl. tetani*. Strain S-17, however, possessed the common heat-stable antigenic factor IV and a specific factor VI for itself. Based upon the reactions of the heat-stable antigen, strain S-17 was considered representative of a species distinct from *Cl. tetani*, *Cl. sporogenes*, *Cl. parabotulinum*, and *Cl. histolyticum*.

It was proposed that the serological position of species of proteolytic clostridia be based upon the reactions of the heat-stable antigen. Since the heat-stable antigenic factors were assigned Roman numerals, it was proposed to change the group designations I, II and III to A, B and C, respectively. The *Cl. tetani* strains were placed in group D and assigned antigenic formulas corresponding to the nine types. *Cl. sporogenes* S-17 was assigned to group E.

STUDIES ON THE TOXICITY OF TYPHUS RICKETTSIAE

III. OBSERVATIONS ON THE MECHANISM OF TOXIC DEATH IN WHITE MICE AND WHITE RATS

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The lethal toxic effect of typhus rickettsiae for white mice is well known but the basic mechanism underlying this phenomenon has not been elucidated. The original observation by Gildemeister and Haagen[1] that white mice died several hours after inoculation with murine typhus rickettsiae was confirmed and found applicable to the rickettsiae of epidemic typhus by Bengtson, Topping and Henderson.[2] Subsequently, certain strains of scrub typhus were found to possess similar properties.[3] Very recently, Price[4] and Bell and Pickens[5] have independently demonstrated that rickettsial strains of Rocky Mountain spotted fever are also toxic for mice. All of the above workers noted that the lethal toxic effect of rickettsiae for mice was intimately associated with viability of the microorganisms and could be neutralized by specific antiserum. Cox[6] has reviewed the practical application of the mouse toxicity test for titration of rickettsial suspensions, vaccine assay, and certain immunologic tests.

In this laboratory it was found[7] that white rats were susceptible to the toxic effect of typhus rickettsiae and that pathologic changes associated with rickettsial toxicity occurred in both white rats and white mice.[8] Mice appear normal for 30 minutes or more after injection with lethal toxic doses of viable typhus rickettsiae. Soon thereafter the following manifestations are observed: the animals huddle together; they become less active although they may exhibit sporadic sudden convulsive movements; respiration becomes labored and

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1. Von Gildemeister, E. and Haagen, E. 1940, Fleckfieberstudien. I. Mitteilung: Nachweis eines Toxins in Rickettsien-Eikulturen (*R. mooseri*). Deutsche med. Wchnsch. **66**: 878–880.
2. Bengtson, I. A., Topping, N. H. and Henderson, R. G. 1945, Studies of typhus fever. Epidemic typhus: demonstration of a substance lethal for mice in the yolk sac of eggs infected with *Rickettsia prowazeki*. Nat'l. Inst. Health Bull. **183**: 25–29.
3. Smadel, J. E., Jackson, E. B., Bennett, B. L. and Rights, F. L. 1946, A toxic substance associated with the Gilliam strain of *R. orientalis*. Proc. Soc. Exper. Biol. & Med. **62**: 138–140.
4. Price, W. H. 1953, A quantitative analysis of the factors involved in the variations in virulence of rickettsiae. Science, **118**: 49–52.
5. Bell, E. J. and Pickens, E. G. 1953, A toxic substance associated with the Rickettsias of the Spotted Fever Group. J. Immunol. **70**: 461–472.
6. Cox, H. R. 1951, Some recent advances and current problems in the field of rickettsial diseases. Arch. f. d. gesamte Virus forschung, **4**: 518–533.
7. Neva, F. A. and Snyder, J. C. 1952, Studies on the toxicity of rickettsiae. I. Susceptibility of the white rat, with a note on pathologic changes. J. Infect. Dis. **91**: 72–78.
8. Parker, F., Jr. and Neva, F. A. 1954, Studies on the toxicity of typhus rickettsiae. II. Pathologic findings in white rats and white mice. Am. J. Path. **30**: 215–237.

cyanosis may be noted; finally, prostration and death ensue within 1.5 to 6.0 hours. With smaller doses of rickettsiae, toxic death may occur up to 18 hours after injection.

The report of Fox and Clarke[9] included an attempt to elucidate the mechanism of the toxic action of typhus rickettsiae. These workers noted hemoconcentration and decrease in blood available by heart puncture in mice dying of rickettsial toxic death; hence the presence of marked vascular permeability was postulated.

It was hoped that the study of physiologic changes associated with rickettsial toxicity in the intact animal might provide some clues as to the basic mechanism involved. This paper presents studies of the altered physiology associated with rickettsial toxicity in white mice and white rats and proposes an explanation for the mechanism based upon these and previous observations.

METHODS

As reported previously,[7] rickettsial suspensions were prepared from pooled yolk sacs of infected embryonated eggs, distributed in aliquots and stored at −70 C in glass-sealed ampules until used. Before injection into animals, infected suspensions were clarified of particulate material by centrifugation at 1000 r.p.m. for 5 minutes and all dilutions were made in sucrose PG buffer solution at pH 7.0.[10] A single pool of each of three rickettsial strains was used for all of the tests reported herein: the Wilmington strain of murine typhus (*Rickettsia mooseri*), the Breinl strain of epidemic typhus (*Rickettsia prowazeki*), and the human avirulent epidemic strain E,[11] which nevertheless possesses toxicity for mice comparable to the Breinl strain of epidemic typhus.

Animals used throughout were white Swiss mice of both sexes weighing 10 to 20 g, and white male rats of the Harvard Biological Laboratory (Hisaw strain) weighing 130 to 220 g. For the blood pressure experiments larger white rats of both sexes were used with weights of 400 to 500 g. Animals were injected intravenously as previously described[7] in volumes of 0.25 ml for mice and 1.0 ml for rats, unless noted to the contrary. Rickettsial toxin titrations were done in mice by the intravenous injection of half-log dilutions into four animals at each dilution, such as 10^{-1}, $10^{-1.5}$, $10^{-2.0}$, etc., and the LD_{50} was calculated by the method of Reed and Muench.[12] Inactivation of rickettsial suspensions by heat was carried out in a water bath at 56 to 60 C for 30 minutes.

Chemicals and drugs administered to mice in testing their effect upon rickettsial toxicity were injected intra-abdominally with the animal under light ether anesthesia. Experiments not reported in this paper indicated clearly that a few minutes of light ether anesthesia and the intra-abdominal injection of 0.25 ml or less of saline solution as a control material, had no significant influence upon the rickettsial toxin LD_{50}.

Blood for specific gravity measurements was obtained in both white mice and white rats by cardiac puncture of the animal while under light ether anesthesia. In mice, blood coagulation was prevented by administration of 0.1 ml commercial heparin solution (Abbott) intra-abdominally no earlier than 20 minutes after injection of the rickettsial suspension or at least 10 minutes before bleeding. The possible effects of heparin upon the toxic phenomenon are considered in the text. Rats, however, were not heparinized to prevent coagulation of blood specimens. Instead, a standard volume of 0.5 ml of blood was drawn with a dry 23-gauge needle and a 1-ml tuberclin syringe previously rinsed with liquid heparin. Blood specimens were stored in the refrigerator for no longer than 3 hours after being drawn before they were used for measurements of specific gravity.

Whole blood and plasma specific gravities were measured by the falling drop method of Barbour[13] in xylene-brombenzene mixtures. Standard refer-

9. Clarke, D. H. and Fox, J. P. 1948, The phenomenon of in vitro hemolysis produced by the rickettsiae of typhus fever, with a note on the mechanism of rickettsial toxicity in mice. J. Exper. Med. **88**: 25–41.
10. Bovarnick, M. R., Miller, J. C. and Snyder, J. C. 1950, The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. J. Bact. **59**: 509–522.
11. Gallardo, F. P. and Fox, J. P. 1948, Infection and immunization of laboratory animals with *Rickettsia prowazeki* of reduced pathogenicity, strain E. Am. J. Hyg. **48**: 6–21.
12. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**: 493–497.
13. Barbour, H. G. and Hamilton, W. F. 1926, The falling drop method for determining specific gravity. J. Biol. Chem. **69**: 625–640.

TABLE 1.—*Reproducibility of rickettsial toxin titration in white mice.*

Number of titrations	Rickettsial strain	Ratio of survivors to total number injected, cumulative, at dilutions				Mean LD_{50}*	Range LD_{50}	Standard deviation of LD_{50}§
		$10^{-1.0}$	$10^{-1.5}$	$10^{-2.0}$	$10^{-2.5}$			
20	Murine (Wilmington)	0/18	4/85†	13/83	76/77‡	2.18	1.83–2.25	0.11
12	Epidemic (strain E)	0/30	0/51	27/52	32/32	2.00	1.75–2.17	0.18
5	Epidemic (Breinl)		0/19	12/20	20/20	1.93	1.83–2.17	0.14

* Expressed as log of denominator of the dilution when calculated by the Reed-Muench formula.
† In 3 of the 20 titrations there were survivors after injection with $10^{-1.5}$ dilution; 1 of 4, 1 of 6, and 2 of 6 mice injected.
‡ In only one of 20 titrations death resulted from the $10^{-2.5}$ dilution; in 1 of 4 mice injected.
§ At best this value represents a rather crude estimate of standard deviation: only a single dilution, tested in only 4 mice, lay between 100 and 0 percent mortality in each titration.

ence solutions were prepared with K_2SO_4 and distilled water in mercury-calibrated volumetric flasks. Standard specific gravity solutions were stored under mineral oil. The dropping pipette was also calibrated with mercury. Estimation of whole blood and plasma specific gravity was then made from an alignment chart with temperature correction on the basis of falling time values measured with a stop watch.

Direct and continuous measurement of mean arterial blood pressure in the rat was accomplished by cannulation of a femoral artery with a PE100 polyethylene tube which was connected to a mercury manometer. The artery was cannulated, the polyethylene tube sutured firmly in place, and the wound closed while the rat was under ether anesthesia. Saline solution containing heparin comprised the fluid junction for the manometer, and 5 or 10 minutes after cannulation an additional 0.3 ml of liquid heparin was injected via the cannula. The rat was placed in a cylindrical screen cage which allowed the animal only slight forward and backward movement. Ends of the cylindrical screen cage were closed with only the cannula tubing and the tail protruding. The cage was securely taped on a table beside the manometer and the animal was allowed to recover from anesthesia. The manometer was equipped with an ink writing stylet so that continuous measurement of arterial blood pressure was recorded on a slowly moving paper. Rickettsial suspensions were injected into a tail vein after the rat had been cannulated. With this method blood pressures could be recorded without anesthesia for periods up to 6 hours. The manometer and recording device did not have sufficient sensitivity to record pulse pressures but the dampened oscillation still permitted a check on the patency of the system. A three-way stopcock between manometer and tubing allowed saline solution to be injected to flush the cannula and to withdraw blood specimens.

EXPERIMENTAL

Reproducibility of the titration for rickettsial toxicity in white mice.—Over a period of one year a pool of each of three separate strains of typhus rickettsiae was titrated in white mice for lethal toxicity at various times but always under standard conditions. All of the tests including dilution of rickettsial suspensions were done by the same worker. In table 1 are summarized the results of 20 titrations with a single pool of murine typhus rickettsiae, 12 titrations with one pool of epidemic typhus strain E, and 5 titrations with the Breinl strain of epidemic typhus. It is evident that for a biologic test the titration of rickettsial toxicity in mice has a satisfactory degree of reproducibility.

It should be noted that the method used provided either none, or only four animals, depending upon the end point, at a single dilution between 100 and 0 percent mortality. Consequently, an accurate measure of LD_{50} and a standard deviation derived therefrom may not be justified in the strict statistical sense. However, the titration for rickettsial toxicity at half-log dilution intervals represents a standard procedure that was adopted after consideration of the possible sources of technical error inherent in the test. The data in table 1, therefore, show what may be expected in consistency of the test as routinely employed. Upon the basis of our previous experience, as well as the results presented here, differences up to one-half log in the toxic LD_{50} for a given rickettsial preparation have been considered due to chance for the test as performed in this laboratory.

TABLE 2.—*Whole blood specific gravity values in white mice under various conditions.*

Group	I No injection controls	II Normal yolk sac	III Toxic dose in immune mice	IV Lethal toxic dose	V Small sublethal toxic dose	VI Larger sublethal toxic dose
Time after injection	No injection	75 to 100 min.	75 to 100 min.	75 to 100 min.	75 to 100 min.	100 to 135 min.
Whole blood specific gravity values	1.053	1.052	1.055	1.059	1.053	1.059
	1.051	1.052	1.056	1.060	1.052	1.058
	1.052	1.046	1.055	1.069	1.053	1.058
	1.052	1.044	1.056	1.060	1.055	1.056
	1.052	1.055	1.052	1.074	1.054	1.058
	1.049	1.052	1.057	1.072	1.056	1.058
	1.051	1.052	1.053	1.057	1.049	1.057
	1.053	1.054	1.056	1.063	1.055	1.058
	1.054	1.053	1.057	1.073	1.052	1.049
	1.050	1.056	1.059	1.068	1.052	1.056
	1.053		1.058	1.071		1.055
	1.050			1.072		1.052
	1.049			1.068		1.059
	1.054			1.060		1.057
	1.051					1.059
	1.051					1.058
	1.055					1.055
						1.056
						1.061
						1.055
						1.053
						1.055
Mean	1.052	1.052	1.056	1.066	1.053	1.056
Standard deviation	0.0036	0.0036	0.0046	0.0063	0.0019	0.0041

Group I—Normal mice bled without previous injection.
Group II—Injected intravenously with 0.25 ml of 3.2% normal yolk sac.
Group III—Injected intravenously with 0.25 ml of 3.2% infected yolk sac of murine typhus 23 to 27 days after previous murine typhus infection.
Group IV—Injected intravenously with 0.25 ml of 3.2% infected yolk sac of murine typhus.
Group V—Injected intravenously with 0.25 ml of 0.2% infected yolk sac of murine typhus.
Group VI—Injected intravenously with 0.25 ml of 0.32% infected yolk sac of murine typhus.

Percent yolk sac refers to grams wet weight of whole yolks sacs suspended in 100 ml sucrose PG solution.

Changes in blood specific gravity associated with rickettsial toxicity in white mice and white rats.—The observation of hemoconcentration in mice dying of the rickettsial toxic effect[9] was investigated further by measurements of the whole blood and plasma specific gravity in white rats as well as white mice. Furthermore, the specificity of the findings in mice was assessed by study of the specific gravity values under various conditions, and the use of rats permitted multiple measurements in the same animal. The falling drop technic requires only one drop of whole blood or plasma per determination, but in most instances duplicate falling times were clocekd on each sample.

Findings in white mice.—Control reference values of whole blood and plasma specific gravities were first obtained from 17 normal white mice. Whole blood specific gravity values from these animals are listed under group I in table 2. Measurements were then made in groups of mice 75 to 100 minutes after injection with yolk sac suspensions under various conditions: group II mice received *normal* yolk sac suspension; group III was rendered *immune* to toxic death by infection with murine typhus, and then injected 23 to 27 days later with a suspension of murine typhus rickettsiae which constituted a known lethal toxic dose in susceptible mice; group IV received a *lethal* toxic dose of murine rickettsiae; and group V was injected with a small *sublethal* dose of murine typhus. Group VI animals received a slightly larger *sublethal* dose, of the same murine rickettsial pool given to groups III, IV, and V but were bled for specific gravity values 100 to 135 minutes after injection. It should be noted that all mice except control group I were injected by the intravenous route, and all

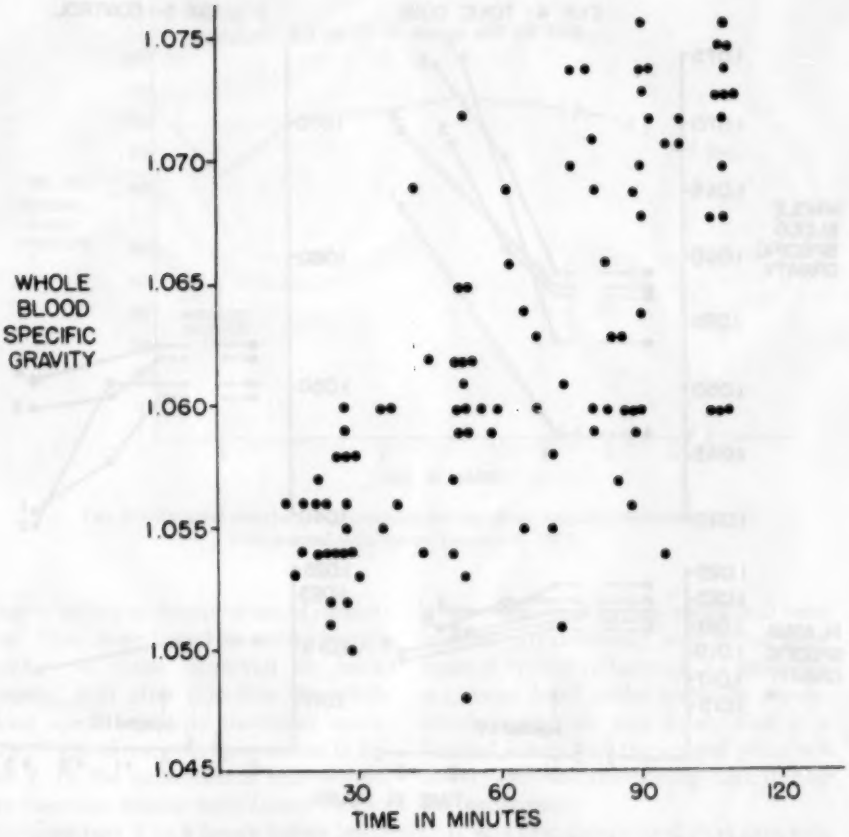

FIG. 1.—Accumulated whole blood specific gravity values in white mice after intravenous injection of a lethal toxic dose of rickettsiae. Each mouse was injected intravenously with 0.25 ml of 3% yolk sac suspension of the same pool of murine typhus rickettsiae.

received the same volume of inoculum, 0.25 ml. Aliquots of a single pool of murine typhus were used for groups III, IV, V and VI. Results are tabulated in table 2.

The mean whole blood specific gravity in control mice which received no injection showed no significant difference from mice which received normal yolk sac. These values, however, were in obvious contrast to the whole blood specific gravities of mice dying from rickettsial toxicity. A slight but significant increase in whole blood specific gravity was found in the group of immune mice given a lethal toxic dose (group III) and in those animals (group VI) bled approximately 2 hours after injection with the larger sublethal dose of typhus rickettsiae.

Values for plasma specific gravities are not tabulated in detail because the range and mean for each group were found to be almost identical (1.016 to 1.023, with means of 1.018 or 1.019 for each group).

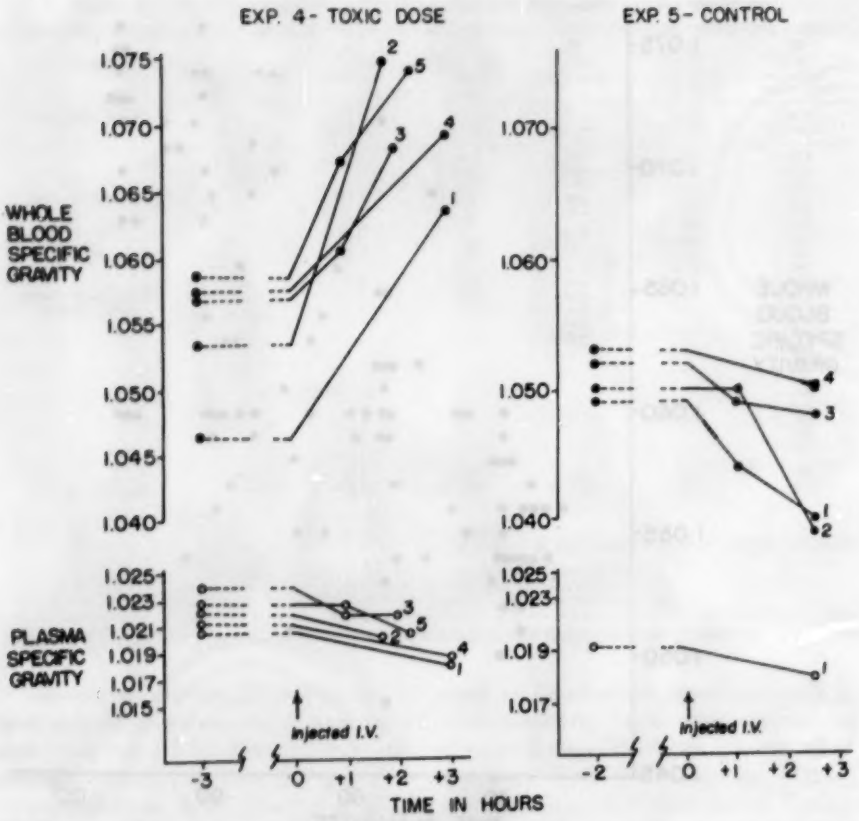

FIG. 2.—Whole blood and plasma specific gravity values in white rats after intravenous injection with lethal toxic dose of epidemic typhus rickettsiae (exp. 4) and with control preparations (exp. 5). In experiment 4 each rat received 1.25 ml of 10% infected yolk sac suspension, which usually produced death in 2 to 4 hours. In experiment 5, rat no. 1 received 1.25 ml of 10% normal yolk sac suspension and no. 2, 3 and 4 were given 1.25 ml of the same infected suspension used in experiment 4 but heated at 56 C for 30 minutes.

In figure 1 are shown whole blood specific gravity values from several experiments in which measurements were taken at varying time intervals following the intravenous injection of a lethal toxic dose of rickettsiae. The same dose of a single murine typhus pool was used in all experiments represented. In addition to the very evident increase in whole blood specific gravity demonstrated in the scatter graph, it is apparent that the upward trend began within 30 to 60 minutes after injection and was not merely a terminal event. On the basis of numerous other experiments with the same toxic dose in animals which were only observed and not bled, death occurred approximately 80 to 120 minutes after injection.

Findings in white rats.—With white rats it was possible to obtain blood specimens from the same animal before and

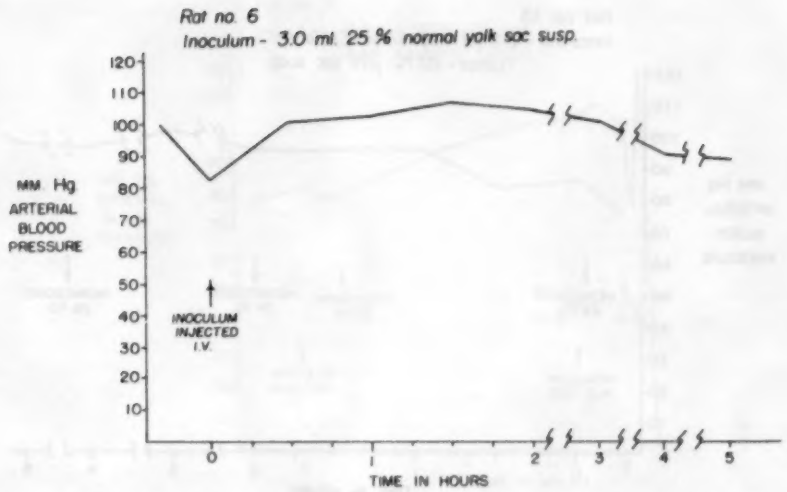

FIG. 3.—Femoral arterial blood pressure tracing of rat injected intravenously with normal yolk sac on January 9, 1953.

after injection with toxic doses of rickettsiae. They were found to show changes similar to those observed in mice, namely, that after injection the whole blood specific gravity increased markedly. Typical responses are shown in figure 2. In the experiments represented, pre-injection bloods were drawn by cardiac puncture 2 to 4 hours before injection. Epidemic typhus rickettsiae were used and the control animals (exp. 5) received either a comparable amount of normal yolk sac suspension or heat killed rickettsiae. The decrease in whole blood specific gravities seen in the control rats and in the plasma values for the toxic rats was attributed to the effect of blood loss from the previous bleeding.

Blood pressure in white rats dying of rickettsial toxicity.—The blood specific gravity findings and the appearance of animals in the terminal stages of rickettsial toxicity suggested a possible correlation of these events with changes in blood pressure. Therefore, continuous records of direct arterial blood pressure levels were made in rats which had been injected intravenously with lethal toxic doses of typhus rickettsiae. As described in greater detail under methods, a polyethylene cannula was firmly tied in a femoral artery and the animal remained in a cylindrical restraining cage during the experiment.

It was first determined that rats subjected to this procedure and injected intravenously with either suspensions of normal yolk sac or of heat-killed rickettsiae maintained their blood pressures at normal levels for as long as 5 hours after injection. Furthermore, withdrawal of 0.8 ml samples of arterial blood via the cannula for hematocrit determinations from a rat injected with heat killed rickettsiae resulted in only a fall in hematocrit but no effect upon blood pressure. These findings are illustrated in figures 3 and 4.

Technically satisfactory experiments of the same kind were performed in 6 rats, all of which were injected with an equal toxic dose of epidemic typhus

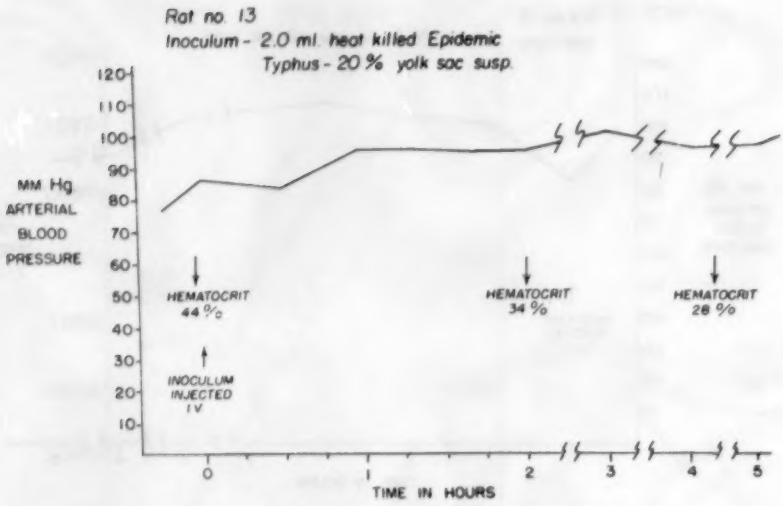

FIG. 4.—Femoral arterial blood pressure tracing of rat injected intravenously with heated typhus rickettsiae on March 2, 1953.

rickettsiae. These animals all showed a consistent pattern of blood pressure response with either no change or a moderately slow rise in pressure within two hours after injection, followed by a precipitous fall in pressure just before death. In every instance death occurred within a period of 2 to 3 hours after injection of rickettsiae, and the terminal fall in blood pressure in all cases occurred only 20 to 30 minutes before death. Representative tracings are re-

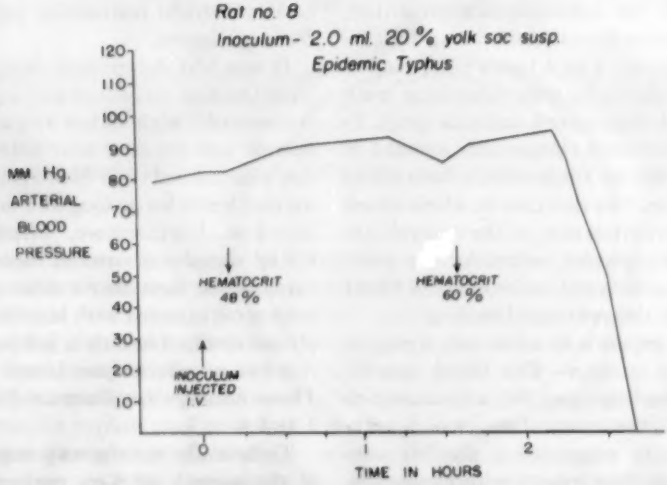

FIG. 5.—Femoral arterial blood pressure tracing and hematocrits of rat injected intravenously with toxic dose of typhus rickettsiae on March 6, 1953.

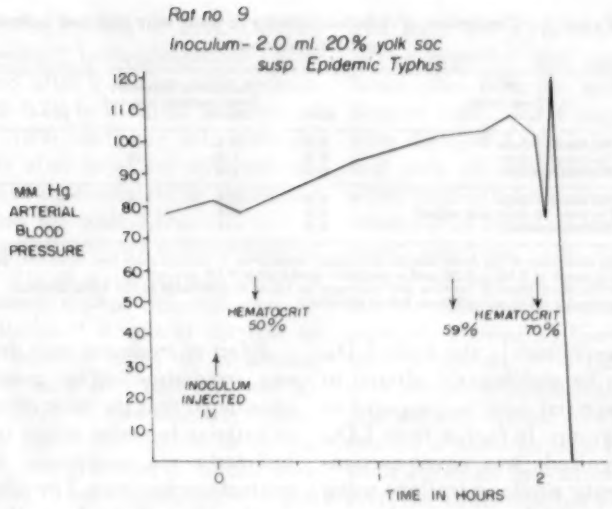

FIG. 6.—Femoral arterial blood pressure tracing and hematocrits of rat injected intravenously with toxic dose of typhus rickettsiae on March 27, 1953.

produced in figures 5 and 6. From two toxic animals hematocrits were determined on arterial blood obtained via the cannula; these showed a rise in hematocrit after injection with rickettsiae.

The hematocrit values in toxic white rats corroborate the blood specific gravity findings. These experiments clearly establish the fact that the hemoconcentration associated with the phenomenon of rickettsial toxicity in rats and mice occurs well before, and independently of, any change in the blood pressure.

Influence of various treatments upon rickettsial toxicity in white mice: effect of water deprivation.—In view of the blood specific gravity changes compatible with hemoconcentration found in toxic mice and rats, hemoconcentration produced by other means was tested for its possible influence upon rickettsial toxicity. In preliminary experiments it was found that 13 to 18 g white mice maintained on dry pellets of feed without water remained active and exhibited no obvious ill effects for as long as 5 to 7 days. Mice deprived of water in this manner for 4 days lost approximately 4 to 5 g of body weight and were found to have a moderate elevation of both whole blood and plasma specific gravities. Several mice tested at random after 4 days without water showed whole blood specific gravities of approximately 1.060 to 1.064 and plasma specific gravities of 1.023 to 1.026.

In one experiment white mice kept without water for 2 and 4 days were compared to normal control mice with respect to the LD_{50} toxicity of a single murine typhus preparation titrated in each group. In another experiment a 4-day period of water deprivation was again chosen, but half of the mice were given 2 ml of saline solution intra-abdominally 1 hour before titration for rickettsial toxicity. The water deprived, saline treated, and a normal group of control mice were then all titrated simultaneously for toxicity with the same murine typhus pool used previously. Results are summarized in table 3.

TABLE 3.—*Comparison of rickettsial toxicity in white mice with and without water deprivation.*

Exp. no.	Treatment	Number of survivors / Total number injected $10^{-1.5}$	$10^{-2.0}$	$10^{-2.5}$	$10^{-3.0}$	LD_{50}*
1	No water 4 days	0/4	0/4	2/6		2.62
	No water 2 days	0/4	1/5	6/6		2.19
	Normal controls	0/4	1/4	4/4		2.16
2	No water 4 days	0/4	0/4	1/4	4/4	2.66
	No water 4 days plus saline†	0/4	0/4	4/4	4/4	2.25
	Normal controls	0/4	1/4	4/4	3/3	2.16

Twenty previous titrations of the same murine rickettsial suspension in normal mice (see table one) gave a mean toxic LD_{50} of 2.18, with a range of 1.83 to 2.25, and a standard deviation of 0.11.
* Calculated by the Reed-Muench formula and expressed as log of denominator of the LD_{50} dilution.
† This group received 2 ml of saline 1 hour before titration.

In both experiments the toxic LD_{50} appeared to be significantly altered in the water deprived mice as compared to the control group. In fact, a toxic LD_{50} of such magnitude was never encountered in twenty similar titrations using the same rickettsial suspension in normal mice. The single experiment suggested furthermore that this increased susceptibility to rickettsial toxicity by dehydration could be corrected by rehydration with saline solution immediately before the test.

Treatment of rickettsial toxicity in mice by administration of fluid.—The alterations in blood specific gravity in mice dying of rickettsial toxicity pointed to the obvious possibility that fluid therapy might prevent or modify the toxic effect of rickettsiae. This was tested in a number of experiments in which saline, 10% glucose, or 5% gelatine solutions were given either intra-abdominally or intravenously, both before and after injection of rickettsiae in amounts up to 1.0 ml per mouse. In some instances one or two of a group of mice given fluids did survive a lethal toxic dose of rickettsiae, but results with fluid therapy were neither consistent nor reproducible. From these experiments it was concluded that rickettsial toxic death in *normal* mice could not be prevented by the parenteral administration of saline, 10% glucose, or 5% gelatine solutions.

Effect of treatment with drugs and X-ray irradiation.—The possibility was considered that the toxic effect of typhus rickettsiae for mice might be mediated indirectly via neurogenic or vascular control mechanisms. The effect of treatment with various drugs therefore was tested by titrating simultaneously the toxic LD_{50} of a murine typhus rickettsial suspension in a treatment and control group of mice. Drugs used in treatment were first screened for an approximation of the lethal dose in white mice and were generally used in maximal tolerated doses. No significant differences of the toxic LD_{50} were noted in treated groups of mice as compared with controls with the following drugs: 0.25 mg atropine sulfate intra-abdominally both before and after injection of rickettsiae; 0.01 mg adrenalin in two doses subcutaneously after administration of rickettsiae; 0.2 mg Benadryl (Parke-Davis) intra-abdominally after injection with rickettsiae; 0.3 to 0.5 mg tetraethyl ammonium chloride intra-abdominally before and after injection of rickettsiae; and 0.05 to 0.12 mg of mecholyl chloride subcutaneously after rickettsiae had been injected. The vasoconstrictors, ergonovine maleate and pitressin failed to show any protective effect in mice given 2 to 3 toxic LD_{50}'s of murine typhus rickettsiae.

Other drugs were also tested. For ex-

ample, 0.5 mg doses of BAL (British Anti-Lewisite)* intra-abdominally both before and after injection with rickettsiae, and 0.10 to 0.25 ml amounts of heparin (1000 units per ml) intra-abdominally after injection with rickettsiae showed no consistent or significant effect upon the toxic LD_{80}.

In view of the known enhancement of susceptibility of white mice to *infection* with *epidemic* typhus rickettsiae by X-ray irradiation,[14] it was of interest to test the possible effect of rickettsial toxicity in mice that had received X-ray irradiation. In two separate experiments no significant difference in the toxic LD_{80} was observed in control mice as compared to mice irradiated with 500 to 700 R from 3 to 4 days prior to the test.

Additional observations on the phenomenon of rickettsial toxicity: plasma sodium and potassium values in white rats dying of rickettsial toxicity.—Blood specimens were obtained from white rats 15 to 30 minutes before injection with known lethal toxic doses of rickettsiae, and again shortly before death of the animals. Similar experiments were done also to test the effect of injection with heat-killed rickettsiae. Plasma sodium and potassium values were determined,† no significant changes in plasma Na+ and K+ from pre-injection values were found.

Leukocyte response associated with rickettsial toxicity.—In two experiments involving 10 white rats, differential counts and total leucocyte counts were done before injection and approximately 2 hours after injection with lethal toxic doses of rickettsiae. Comparable studies were done on 4 rats injected with normal yolk sac suspension, and 4 rats given heat-killed rickettsiae. Blood specimens in all instances were obtained by heart puncture. The total leucocyte counts showed no consistent pattern, but the differential counts in toxic rats regularly showed an increase of 2 to 8 fold in polymorphonuclear leucocytes over the pre-injection values. Two rats injected with normal yolk sac suspension showed a 3-fold increase in polymorphs while 2 animals showed no change. However, the 4 rats injected with heat-killed rickettsiae all exhibited 2- to 6-fold rises in percentage of polymorphonuclears within 3 hours after injection. Too few animals were studied to allow any definite conclusions in regard to specificity of the increase in polymorphocuclear leukocytes associated with rickettsial toxicity.

Attempts to detect kidney damage in white rats dying of rickettsial toxicity.—Since the blood specific gravity changes in toxic mice and rats were compatible with plasma loss, attempts were made to evaluate renal damage with consequent plasma loss via the urine in toxic white rats. The injection of Evan's blue dye, T 1824, intravenously prior to injection with toxic doses of typhus rickettsiae, was employed as a marker for urinary loss of plasma. Passed urine was collected in special screen-bottom cages and in some experiments bladder urine was examined in rats prepared by ligation of the urethra prior to the test. In each experiment control rats were also injected with T 1824. Most experiments showed visually detectable T 1824 in the urine of toxic rats as compared with either none or distinctly less blue color

* Kindly supplied by Hynson, Wescott, Dunning Company.

14. Liu, P. Y., Snyder, J. C. and Enders, J. F. 1941, Fatal infection of irradiated white mice with European typhus by the intra-abdominal route. J. Exper. Med. **73**: 669–679.

† We are indebted to Dr. Nancy Nichols, New England Deaconness Hospital, for the plasma sodium and potassium determinations.

in the urine of control animals. However, in several experiments equal degrees of bluish discoloration of the urine to visual inspection were found in both control and toxic rats. Under these circumstances no definite conclusions were made as to possible renal damage and plasma loss via urine in white rats dying of rickettsial toxicity.

Failure to transfer rickettsial toxicity via tissues or plasma exposed to rickettsiae.—The fact that rickettsial toxicity is intimately associated with the viable microorganisms themselves has been already mentioned. Yet, the possibility might be entertained that the action of rickettsiae upon certain cells or plasma in the mouse or rat is only the first step in a series of events in the toxic reaction.

In one experiment pooled plasma collected 1 to 2.5 hours after injection of 19 mice with a lethal toxic dose of rickettsiae, and then injected intravenously in 0.3 to 0.5 ml amounts into 3 fresh mice failed to produce untoward effects. Thus, under these circumstances, there was no evidence that the toxic effect could be transferred via the plasma of mice dying of rickettsial toxicity.

In another experiment pooled mouse plasma incubated with large numbers of typhus rickettsiae at 37 C for 1 hour and then centrifuged free of rickettsiae failed to produce death or ill effects when injected into fresh mice.

Freshly ground suspensions of normal mouse liver and spleen were incubated for 1 hour at 37 C with murine typhus rickettsiae. The centrifuged rickettsial free supernates of such suspensions also failed to produce death similar to the rickettsial toxic death when injected intravenously into mice. A few of the mice died within 5 minutes after injection but these were attributed to causes other than rickettsial toxicity.

Miscellaneous observations.—The idea was considered that there might be an altered susceptibility to rickettsial toxicity in mice which were reacting to diphtheria toxin. A titration of a rickettsial suspension was performed in control mice and in mice which were midway in the interval between inoculation of 2 LD_{50}'s of diphtheria toxin and death therefrom. The end points were identical in the two sets of mice.

Several experiments were done in an effort to determine whether alloxan induced diabetes in mice might alter response to rickettsial toxin. No consistent changes were observed.

Pretreatment by the oral route with relatively large doses of chlortetracycline or oxytetracycline for 2 to 4 days had no effect on the toxicity titration end points of epidemic typhus in mice.

DISCUSSION

The mechanism of rickettsial toxicity for white mice and rats involves basic relationships between a viable microorganism and its host. With present methods the titration of toxicity constitutes a more reproducible and stable indicator of biologic activity for typhus rickettsiae than infectivity for eggs or for susceptible animals.[15,16] Although rickettsial toxicity and infectivity have hitherto been considered inseparable, it has recently been possible to dissociate the two properties by ultraviolet irradiation.[15] However, irradiated rickettsiae continue to exhibit metabolic activity and hence must still be regarded as viable organisms.

The experiments described herein now make it possible to reconstruct the phenomenon of rickettsial toxicity in terms

15. Allen, E. B., Bovarnick, M. R. and Snyder, J. C. 1954, The effect of irradiation with ultraviolet light on various properties of typhus rickettsiae. J. Bact. **67**: 718–723.
16. Fuller, H. S. 1954, Quantitative comparisons of responses of cotton rats and albino mice to measured doses of epidemic typhus rickettsiae. J. Immunol. **73**: 138–145.

of physiologic changes. It was shown that soon after injection with toxic doses of rickettsiae mice underwent an increase in whole blood specific gravity; it increased progressively and was not merely a terminal event. Control experiments established the specificity of the rise in blood specific gravity as due to the effect of viable rickettsiae, and the validity of these changes was strengthened by their demonstration in rats as well as in mice. Several experiments in rats disclosed an associated increase in hematocrit. Thus, it was evident that a progressively increasing hemoconcentration occurred in mice and rats injected with toxic doses of typhus rickettsiae.

Although several hypothetical possibilities could explain the occurrence of hemoconcentration it is necessary to interpret the observations in the light of all available knowledge concerning the toxic phenomenon. Hemoconcentration could result from an increase in circulating red blood cells, a decrease in circulating fluid portion of the blood, or a combination of both these events. However, examination of rats and mice which have succumbed to rickettsial toxicity shows marked engorgement of the viscera (including the spleen) with blood, rather than a depleted vascular system. We have invariably found a striking decrease in blood available by cardiac puncture in mice and rats shortly before death from rickettsial toxicity as was reported by Clarke and Fox.[9] Although pooling of blood in the viscera of toxic animals might account for a decreased cardiac bleeding volume it would require selective pooling of plasma at the expense of red cells to produce the hemoconcentration which was observed. Therefore, the only tenable explanation for the hemoconcentration is that a reduction in circulating fluid portion of the blood occurs instead of an increase in red blood cells. Finally, since no significant changes in plasma specific gravity values were found in the presence of increased whole blood specific gravities, it follows that the decrease in circulating fluid represented loss of whole plasma rather than loss of water alone.

Continuous blood pressure measurements, without anesthesia, in the intact rat after injection with toxic doses of rickettsiae showed that blood pressure was maintained at or very near normal levels until just before death, when the blood pressure suddenly failed. It should be noted that a hypotensive state could not have caused hemoconcentration since blood pressures remained normal until hemoconcentration had become well established. This would indicate that the animals were capable of adjusting to their fluid loss for some time until a critical stage was reached and compensatory mechanisms failed.

The interpretation of physiologic events which has been discussed above is supported by pathologic findings which have been described[8] in mice and rats in association with rickettsial toxicity. The upper and mid portion of the small bowel was engorged with blood and the lumen of the gut showed the presence of yellowish fluid. Actual hemorrhage of the bowel was often present in rats. No explanation is available as to why gross pathologic findings were confined to the small bowel unless this area is particulary vulnerable to vascular injury. It is of interest that similar changes in the small bowel have been described[17,18] in dogs subjected to ex-

17. Erlanger, J. and Gasser, H. S. 1919, Studies in secondary traumatic shock II. Shock due to mechanical limitation of blood flow. Am. J. Physiol. **49**: 151–173.

18. Werle, J. M., Cosby, R. S. and Wiggers, C. J. 1942, Observations on hemorrhagic hypotension and hemorrhagic shock. Am. J. Physiol. **136**: 401–420.

perimental shock. In our opinion it is likely that in the toxic animals additional plasma loss may have been present diffusely throughout the body which could not be detected grossly or microscopically.

The hepatic lesions of focal necrosis which occurred[8] several days after the administration of sublethal toxic doses of rickettsiae may represent the histologic sequelae of functional injury to cells by viable rickettsiae. Thus, it is possible that plasma loss in rats and mice dying of rickettsial toxicity is related to direct injury of vascular endothelial cells and subsequent altered permeability by large numbers of viable rickettsiae.

The loss of normal vascular reactivity mediated by humoral factors, and subsequent visceral pooling of blood have been emphasized in the concept of experimental shock.[19] If similar factors play a role in the phenomenon of rickettsial toxicity they probably are secondary to other physiologic events which first produce plasma loss and hemoconcentration.

The concept that fluid loss and hemoconcentration constitute the main pathologic physiology of rickettsial toxicity in mice and rats is supported by the experiments which indicated enhanced susceptibility to toxicity in mice deprived of water. Also, this enhanced susceptibility could be corrected by administration of saline immediately prior to injection with rickettsiae. The inability to protect normal animals from toxicity by parenteral administration of saline, glucose, or gelatin solutions does not necessarily invalidate this hypothesis. The problem of fluid treatment was not investigated exhaustively; the amount, rate, or type of fluid replacement may not have been adequate. Furthermore, it is possible that if damage to vascular permeability occurs it may be irreparable to any kind of fluid replacement therapy.

It should be noted that the phenomenon of rickettsial toxicity as exhibited in mice and rats and with which we are concerned is distinctly different from the hemolytic effects of typhus rickettsiae in rabbits reported recently by Paterson *et al.*[20] These workers described an interesting response in rabbits injected intravenously with large doses of murine typhus rickettsiae which was characterized chiefly by in vivo hemolysis of red cells, hyperkaliemia, hypotension, and without hemoconcentration. We have found that blood samples carefully drawn and processed from toxic mice and rats do not show hemolysis. Our findings of normal blood pressure until just before death, normal plasma potassium values, and progressive hemoconcentration are in distinct contrast to the findings in rabbits. In our opinion the sequence of events in rabbits probably represents a manifestation of the hemolytic properties of rickettsiae and may differ considerably from the toxicity for mice and rats, in which no hemolysis is evident.

The failure of various treatments and drugs to influence the rickettsial toxic effect suggests that the mechanism of toxicity is not mediated via indirect physiologic controls. It has already been shown that pretreatment with ACTH[21]

19. Shorr, E. 1951, Etiology and mechanisms of shock. IV. The role of hepatorenal vasoconstrictive factors in experimental shock. U. S. Army Med. Service Grad. School Sym. on shock.

20. Paterson, P. Y., Wisseman, C. L., Jr. and Smadel, J. E. 1954, Studies of rickettsial toxins. I. Role of hemolysis in fatal toxemia of rabbits and rats. J. Immunol. **72:** 12–23.

21. Kass, E. H., Neva, F. A. and Finland, M. 1951, Failure of ACTH to protect against acutely lethal toxins of influenza virus and rickettsiae. Proc. Soc. Exper. Biol. & Med. **76:** 560–563.

or cortisone[22] has no influence upon rickettsial toxicity in mice, so these hormonal factors do not appear to be important in the pathogenesis of this disorder. Finally, the direct action of viable typhus rickettsiae upon the susceptible host appears necessary, since it was not possible to transfer the toxic properties via a humoral route from affected animals. Therefore, it appears reasonable to postulate that the phenomenon of rickettsial toxicity involves direct injury upon a cellular level which is initiated rapidly after administration of viable typhus rickettsiae.

On the basis of the findings presented here and pathologic findings reported previously,[8] the following hypothesis can be advanced for the mechanism of rickettsial toxicity in mice and rats. Large numbers of viable typhus rickettsiae when administered to these animals produce direct injury to cells, particularly to cells of the vascular endothelium. An alteration in capillary permeability produces a rapid loss of plasma into the tissues, particularly those of the small bowel. The animal is able to compensate temporarily for the plasma loss as evidenced by maintenance of normal blood pressure in spite of a progressive hemoconcentration. With continuing plasma loss, however, circulating blood volume diminishes to the point where blood pressure cannot be maintained; and the animal dies after a sudden fall in blood pressure.

22. Jackson, E. B. and Smadel, J. E. 1951, The effects of cortisone and ACTH on toxins of rickettsiae and Salmonella typhosa, J. Immunol. 66: 621–625.

SUMMARY

Further studies on the toxicity of typhus rickettsiae for white mice and white rats have clarified the pathologic physiology which accompanies the phenomenon. Whole blood and plasma specific gravity measurements in both mice and rats disclosed that a progressive hemoconcentration began very soon after injection with a lethal toxic dose of rickettsiae. Appropriate control studies established the specificity of these findings. In rats, direct and continuous measurement of arterial blood pressure showed that, despite a progressive hemoconcentration, the blood pressure was maintained at or near normal levels until shortly before death occurred. These events are interpreted as evidence that large numbers of viable typhus rickettsiae produce direct injury to cells, especially of the vascular endothelium.

ABSENCE OF AMINO ACID INCORPORATION INTO ANTIBODY DURING THE INDUCTION PERIOD

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Remarkably little attention has been paid to the nature of the induction period in the current hypotheses of antibody formation. The one direct study of antibody synthesis during the induction period known to us is that of Green and Anker (1954). These authors first showed that the antibody concentration of carboxyl labeled C^{14}-glycine depended upon the isotope concentration of the nonprotein glycine rather than upon the isotope glycine in the serum proteins. They then administered amino acids labeled with different isotopes at different times during the same secondary response to ovalbumin in rabbits. Thus after a second injection of antigen in a rabbit, they gave C^{14} at 10 hours, N^{15} at 41 hours and H^3 at 89 hours. The induction period lasted about 3 days, and peak titer occurred on the 5th day. They found that both C^{14} and N^{15}, which were administered during the induction period, appeared in the antibody on the 5th day. Furthermore, by comparison with the serum protein labels in this animal and in a second animal administered C^{14}- and N^{15}-labeled amino acids, they calculated that 31% of the antibody protein was synthesized during the first 60 hours after the injection of antigen, i.e., the approximate duration of the induction period, whereas the remaining 69% was synthesized in the following 72 hours during the rise of serum antibody. The discrepancy between the interpretation of these and our results will be considered in the discussion.

In the present work, the synthesis of antibody during the induction period and during the rise of serum antibody was studied by dividing the immune process between two rabbits at about the end of the induction period. This division was effected by transferring spleen mince from a donor rabbit at the end of the induction period following a second injection of bovine serum albumin (BSA) to a normal recipient rabbit (cf. Deutsch, 1899). Such donors or recipients were administered yeast hydrolysate containing amino acids labeled with S^{35}. The antibody formed in the recipients (and in some donors after removal of the spleen) was then studied for radioactivity. A preliminary account by us (1955) has been published.

MATERIALS AND METHODS

Spleen mince transfer.—The secondary response of rabbits to bovine serum albumin (BSA supplied by the Armour Laboratories) was used throughout the present investigation because the induction period and antibody rise to peak titer were each shorter and more clearly demarcated than the initial response and the initial response was used to select good precipitin formers. The primary and the anamnestic responses were induced about a month apart by the intravenous injection of 30 mg BSA/ml 0.85% NaCl/kg rabbit.

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A good primary response was determined by the rate of disappearance of BSA labeled with I^{131}. Thus, rabbits were initially injected with BSA trace-labeled with I^{131} and were presumed to be early and rapid antibody formers when the disappearance of I^{131} from their serum was markedly accelerated between the 8th and 9th day (see Talmage et al, 1951). All traces of iodine label had disappeared from the serum by the time of the second injection of BSA and did not, therefore, interfere with later determinations of S^{35} radioactivity.

Three days after the second injection of a donor rabbit (counting the day of the second injection as 0 day), at a time which usually just preceded the appearance of precipitating antibody in the serum, the spleen was removed, weighed, minced by one up and down excursion in a Teflon tissue grinder in cold Krebs calcium-free phosphate saline with 0.1% added glucose (pH 7.4). The mince was put through a 100 mesh sieve and injected intravenously into one or more recipients either with or without washing. About four-fifths of the original spleen passed through the sieve. The recipients of the transferred spleen mince showed an early rise of serum-precipitating antibody with a peak titer on the 2nd day. The material was washed by suspending it in 15 ml of the cold buffered solution, centrifuging at 1500 r.p.m. for 10 minutes, and after discarding the supernatant, resuspending the sediment in the cold buffered solution. The in vitro interval was 6 to 8 minutes without washing and 18 to 25 minutes with washing. The approximate amounts of spleen mince injected per kg recipient rabbit are given in the tables. In general, when the spleen of a donor was small, i.e, less than 1.5 g, the mince from the entire spleen was injected intravenously into a single recipient. When it was approximately 2 g, the mince was divided between two recipients, and, in one case when it weighed 4 g, it was divided among 3 recipients. Embolic accidents were frequent whenever much more than 1 g of splenic mince was injected intravenously into a rabbit.

For convenience, all donors injected with BSA and supplying spleen mince or serum to recipients are numbered from 1 to 14. All recipients of splenic mince or serum bear the number of their donor with an A or B suffix. The donor rabbits weighed between 2.5 and 3.2 kg and the recipients between 1.8 and 2.5 kg. They were obtained from Mr. Harold Swift's Lakeside Farm and were snuffles-free.

Variable but adequate amounts of antibody for study were formed in 23 recipients after receiving spleen mince from 13 donors. No detectable antibody was formed, however, in 7 recipients receiving spleen mince from 4 other donors.

S^{35}-labeled amino acids.—The labeled amino acids were prepared by Abbott Laboratories and consisted of an acid hydrolysate of yeast to which was added unlabeled tryptophane in approximately the amount lost by the acid hydrolysis. They were always injected into the rabbits intravenously.

Specific activity of serum protein.—Serums were divided into two groups: (1) serums from animals which did not receive S^{35} amino acids directly, e.g., recipients of splenic mince from labeled donors, and (2) the remainder of the serums, i.e., from animals that received S^{35} amino acids directly and which were accordingly labeled to a much higher degree. In group 1, 0.2 ml of each serum was precipitated with 0.8 ml water and 1 ml 20% trichloroacetic acid (TCA). The precipitate after washing with 1 ml water was dissolved in 3 ml 0.04 *N* NaOH. It was then plated in duplicate in one ml amounts for radioassay and 0.5 ml of a 1:5 dilution with saline was assayed for protein nitrogen (N). In group 2, 0.05 ml of each serum was precipitated with 0.95 ml water and 1 ml 20% TCA. The precipitate after washing with 0.5 ml water was dissolved in 5 ml 0.04 *N* NaOH and was then plated in duplicate in 0.5 ml amounts for radioassay and a 0.5 ml sample was used for a protein determination.

Protein determination.—Protein N was determined by a slight modification of the Folin-Cicolteau procedure as adapted by Lowry et al (1951).

Specific activity of antibody.—BSA was labeled with I^{131} and preliminary and final I*BSA precipitation tests were done on each serum according to procedures previously described (Talmage and Maurer, 1953; Talmage et al, 1954). Samples of each serum to be analyzed were mixed with BSA in the region of slight antigen excess, which was estimated from the final I*BSA precipitation test. The mixtures were incubated at 37 C for 30 minutes and left in the refrigerator 4 days. The precipitates were then centrifuged, washed twice in the cold with 0.85% NaCl and dissolved in 1.2 ml of 0.04 *N* NaOH. Of this material, 0.5 ml aliquots were used for protein and radioactivity determinations.

As a control for labeled complement and nonspecific serum factors carried down with the immune precipitate, aliquots of 25 μg of anti-EA N and 2.5 μg of EA (egg albumin) N were added to aliquots (0.5 to 3 ml) of anti-BSA serum. Precipitates were treated as above, except that the entire precipitate after dissolving in 0.5 ml of 0.04 *N* NaOH was transferred to a planchet and counted.

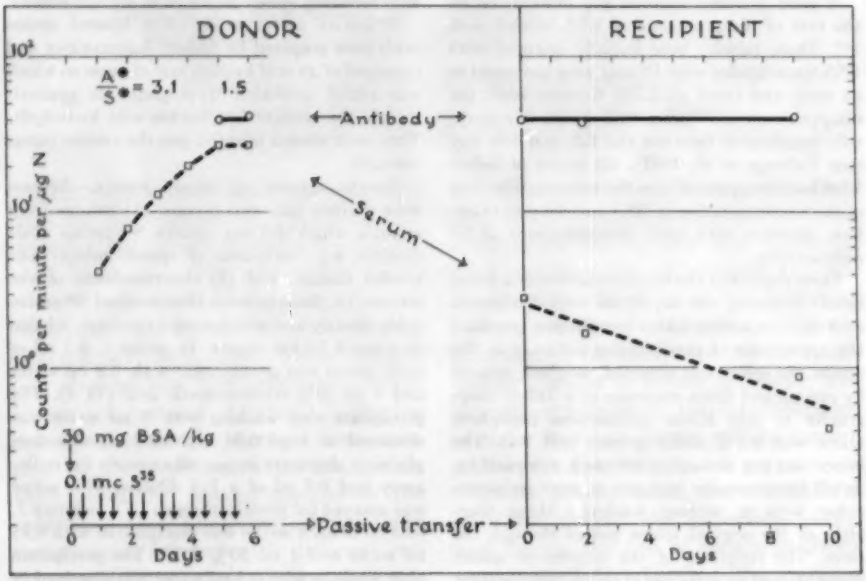


FIG. 1.—Relation of specific radioactivity of antibody protein N to the specific activity of serum protein N(A^*/S^*) in a labeled donor and after passive transfer of its serum to an unlabeled recipient. Note that A^*/S^* is 3.1 on the 3rd day and 1.5 on the 5th and 6th days in the donor.

Decomplementation before analysis of antibody for specific activity.—The serums obtained from recipients given spleen mince from labeled donors were used undecomplemented.

The serums from labeled recipients 9A, 9B, 10A and 10B were decomplemented once by the addition of 50 μg anti-EA N per ml of serum and 5 μg EA N. After incubation at 37 C for 30 minutes, the mixtures were left overnight in the refrigerator. The EA anti-EA precipitates after centrifugation were then discarded. The serums from rabbit 13A were decomplemented 3 times by the successive addition of the same quantities of EA and anti-EA with the same subsequent treatment as just described. This procedure was considered desirable to remove as much labeled complement as possible. This rabbit was a control which received one injection of unlabeled antibody and serial injections of S^{35} amino acids.

Radioassay.—The NaOH protein solutions were placed on oxidized copper planchets within an area of 10 $cm.^2$ Radioactivity was determined in a gas flow internal Geiger counter.* In all cases duplicate radioassays were made and calculations were based on a total of at least 1280 counts. Frequently, a further radioassay was made with another counter on other samples. When necessary, the experimental counts were corrected for resolving loss and self absorption from empirical curves. Corrections for resolving loss were not necessary in counts of 2000/minute or under. At the approximate high limit of the samples (6000 counts/minute), the correction was about 5%. In none of the determinations of the specific radioactivity of antibody N was there enough material (0.1 to 0.15 mg/cm^2 planchet) to give appreciable self absorption. The same was true for determinations of the specific activity of serum N except in a few instances of very low activity. Radioactivity of proteins is expressed throughout the present paper as counts/minute/μg protein N.

* Most determinations were made with model 200A counters made by the Packard Instrument Co.

EXPERIMENTAL RESULTS

Stability of the S^{35} label.—The stability of the isotopic label was tested by the passive transfer of antibody from a labeled donor to a normal recipient and by the reverse experiment of the

TABLE 1.—*Comparison of complement and antibody in the serum of rabbit 13A after 3 decomplementations. This rabbit received unlabeled antibody and 1.4 mc S^{35} during 7 days.*

Day after passive transfer	Complement			Anti-BSA antibody		
	Original serum	Decomplemented serum†		Decomplemented serum†		
	μg N per ml serum	Counts‡ per minute per μg N	Counts‡ per minute per ml serum	μg N per ml serum	Counts per minute per μg N	Counts per minute per ml serum
2	8.5	64.8	70.7	72.3	1.4	97.6
5	8.3	60.3	56.7	43.0	2.2	92.4
7	6.5	55.9	54.7	37.9	1.4	52.4

† Three decomplementations with 50 μg anti-EA and 5 μg EA.
‡ Determined by the radioactivity precipitated from the serum with the addition of 25 μg anti-EA and 2.5 μg EA.

passive transfer of unlabeled antibody to a labeled recipient.

The results of transferring labeled antibody to an unlabeled recipient are given in figure 1. Donor rabbit 11 on the 5th and 6th days showed a specific radioactivity of approximately 40.6 counts/μg antibody protein N. When this antibody was transferred to the recipient, the specific activity remained approximately the same through the 10th day of the passive immunization. In considering the donor and recipient, it should be pointed out that the serum proteins had reached a specific activity of approximately 27 counts/μg protein N at the time of transfer. As a result of dilution by the serum proteins of the recipient, this count immediately dropped to 2.8 counts/μg protein N. Its half disappearance time approximated 5 days. The loss of specific radioactivity was due to the formation of serum proteins in the recipient to replace metabolic decay. No such dilution or disappearance occurred in the specific activity of the antibody because the recipient neither had antibody at the time of transfer nor made antibody later. These results indicate clearly that for a considerable time there is no appreciable loss of the labeled sulfur atom from the persisting antibody.

The reverse transfer was made by injecting the nonimmunized recipient 13A intravenously with 11.5 ml of a high titered unlabeled anti-BSA serum. This rabbit contained 142 μg antibody N/ml serum 10 minutes later. It then received 0.2 mc S^{35} immediately after the injection of serum and 12 subsequent injections of 0.1 mc S^{35} at approximately 12-hour intervals through the succeeding 6 days. Radioassays on the 2nd, 5th and 7th days gave a specific activity of 1.4, 2.2 and 1.4 counts/minute/μg antibody N as contrasted with counts of 36, 41 and 49 for serum protein N on the same days. These values indicate that the passively transferred antibody did not acquire an appreciable labeling with S^{35}. Actual radioactivity counts of the antibody per ml serum were 97, 92 and 52 per minute on the 2nd, 5th and 7th days, respectively (table 1). Complement analyses on control samples of the same serums indicated that, even after decomplementation 3 times with EA and anti-EA (see *Materials and Methods*), about 1 μg complement per ml serum remained and alone gave 70.7, 56.7 and 54.7 counts/minute/ml serum on the same days, respectively (table 1). Thus, on the 7th day, complement accounted for the entire apparent specific antibody activity. That it did not on the 2nd and 5th days is probably due to the fact that a fixed amount of EA and anti-EA were used for the complement analysis of each serum, whereas the amount of anti-BSA was decreasing. On the 7th day, the ability to bind complement was about equal in the two antigen antibody systems,

TABLE 2.—*Specific radioactivity of serum and antibody protein N following S^{35}-labeling of donor.*

Donor					Recipient					
			Antibody					Antibody		
Rabbit no.	Mc S^{35} administered	Serum count per μg N, day 3	μg N per ml serum, day 5	Count per minute per μg N, day 5	Rabbit no.	Gram donor spleen per kg recipient	Serum count per minute per μg N, day 2	μg N per ml serum	Count per minute per μg N	Percent labeled
1	0.2×6	30	987	60	1A	0.42	0.11	11.3	0.7	1.2
					1B	0.46	0.13	15.8	0.5	0.8
2	0.5×6	71	2625	102	2A	0.58	0.11	26.4	3.6	3.5
					2B	0.68	0.14	23.8	4.0	3.9
3	0.5×6	78	†	156‡	3A	0.33	0.08	10.0	1.2	0.8
4	1.0×6	183	†	376‡	4A	0.27	0.18	2.2	3.1	0.8
					4B	0.33	0.13	2.9	2.4	0.7
5	1.0×6	143	†	286‡	5A	0.21	0.17	11.0	4.5	1.6
					5B	0.21	0.19	2.2	16.0	5.6
6	2:day 2.25	70	†	210§	6A§§	0.54	0.02	19.0	0.54	0.26
					6B§§	0.46	0.004	15.0	0.48	0.23
14	0.1×6	16	251	53	14A§§	0.44	0.02	6.6	0.15	0.29
					14B§§	0.47	0.01	9.6	0.15	0.29

† Killed on 3rd day.
‡ Estimated value at 2 times the serum specific activity on day 3.
§ Estimated value at 3 times the serum specific activity on day 5.
§§ Spleen mince washed once.

whereas the anti-BSA was more efficient at the earlier intervals due to the greater amount of precipitate.

S^{35}-labeled donors and unlabeled recipients.—The transfer of splenic materials from 7 S^{35} labeled donors into 13 unlabeled recipients resulted in the formation of antibody with a very low specific radioactivity (table 2, last 2 columns). In 8 recipients, the spleen mince was injected as rapidly as possible into the recipient without washing. Although this procedure entailed the injection of small amounts of donor serum, it was done because the cells of similar spleen mince transfers during hemolysin production progressively lost the ability to confer antibody-forming power on the recipient as the time outside of the body was prolonged. In donors 6 and 14, the splenic materials were washed and gave the lowest radioactivity. Interpretation of the results from donor 6 but not 14 was complicated by the fact that donor 6 only received one injection of S^{35} and its specific antibody radioactivity was only roughly estimated. The results are further explained in the following paragraphs.

Probably the most accurate method of estimating the percentage labeling of antibody in the recipient is that employed for labeled donors 1, 2 and 14. Each donor was labeled with 6 injections of S^{35} yeast hydrolysate at approximately 12-hour intervals for 3 days beginning immediately after the antigen injection. On the 3rd day, the spleen was removed, and the mince from each spleen was divided between two recipients. Labeling of the donors at the same rate was then continued through the 5th day. This was done to get the specific radioactivity of the antibody produced at this level of S^{35} administration. The level of specific radioactivity would not be expected to change under these conditions because antibody was being formed during a constant dosage schedule of labeled amino acids. Had all of the antibody appearing in the recipients been labeled in the donor in precursor form, the radioactivity of the recipient antibody should be identical with that of the donor. The ratio of specific antibody activity in the recipient to that in the donor gives the percentage of antibody in the recipient

TABLE 3.—*The effect of water lysis of labeled spleen on the transfer of labeled antibody.*

Donor					Recipient							
									Antibody			
Rabbit no.	Mc S^{35} administered	Serum count per μg N	Antibody count per μg N (estimated)	Treatment of donor spleen	Rabbit no.	Gram donor spleen per kg recipient	Mc S^{35} administered	Serum count per minute per μg N	μg N per ml serum	Count per minute per μg N	Percent labeled	A*/S*
3	0.5×6†	78	156	Minced H_2O lysed	3A	0.33	None	0.08	10.9	1.2	0.8	
					3B‡	0.26	None	0.07	5.0	0.01	0	
8	None			Minced		0.48						
					8A	0.57	0.4†	18.0	7.1	27		1.5

† Donor 3 received 0.5 mc S^{35} at 12-hour intervals beginning immediately after the injection of antigen and recipient 8A received 0.4 mc S^{35} in 1 injection immediately after receiving the spleen mince.
‡ Recipient 3B received half the water lysed spleen from labeled donor 3 and half the spleen mince treated regularly from unlabeled donor 8.

which was labeled during the induction period in the donor. There was no possibility that the specific antibody activity in the recipient was lessened by a primary antibody response to antigen transferred in the spleen mince because a primary response does not begin until the 8th day or later.

The specific radioactivity of antibody in donor 1 was 60 counts/minute /μg N, whereas the specific activities of recipients 1A and 1B were 0.7 and 0.5 counts, i.e., labelings of 1.2% and 0.8%, respectively. No precipitating antibody could be detected in the donor serum on the day of splenic transfer although it contained coprecipitating antibody. The serums of recipients 2A and 2B possessed a higher radioactivity than recipients 1A and 2B, i.e., labelings of 3.5 and 3.9 % respectively. These findings may probably be attributed to the passive transfer of a small amount of precipitating antibody in the splenic mince from donor 2 because the latter showed 81 μg precipitating antibody protein N/ml at the time of splenic transfer on the 3rd day.

When donors were given repeated doses of S^{35}-labeled amino acids, and were sacrificed on the 3rd day at the time of splenic transfer, their antibody labeling on the 5th day was approximated by multiplying the specific activity of the serum proteins on the 3rd day by a factor of 2. The rationale for this procedure is shown in figure 1. Administration of 0.1 mc at 12-hour intervals to donor 11 resulted in a mean count of 39.8/minute/μg N for the antibody on the 5th and 6th day. This figure was 3.1 times the specific activity of the serum on the 3rd day. In other similar experiments, antibody activities on the 5th day were 3.3, 2 and 1.5 times the specific activities of the serums on the 3rd day. The factor of 2 was, therefore, lower than 2.5, the mean of the 4 ratios.

Using this approximation for corresponding donors, data from the 4 recipients, 3A, 4A, 4B and 5A, showed such low specific radioactivities for antibody N that only 0.7 to 1.6% of the antibody appeared to be labeled in the donor during the induction period. Data from recipient 5B indicated that 5.5% of the antibody was labeled in the donor. This high figure, we believe, is simply an expression of a low production of antibody (2.2 μg/ml as contrasted with 11 μg/ml in its companion recipient 5A). Both recipients, it should be pointed out, received about the same amount of spleen mince (table 2) from the same donor and, therefore, received passively about the same amount of labeled coprecipitating antibody.

A single dose of 2 mc S^{35} was given to donor 6 18 hours before the splenic transfer. At this time, the serum specific

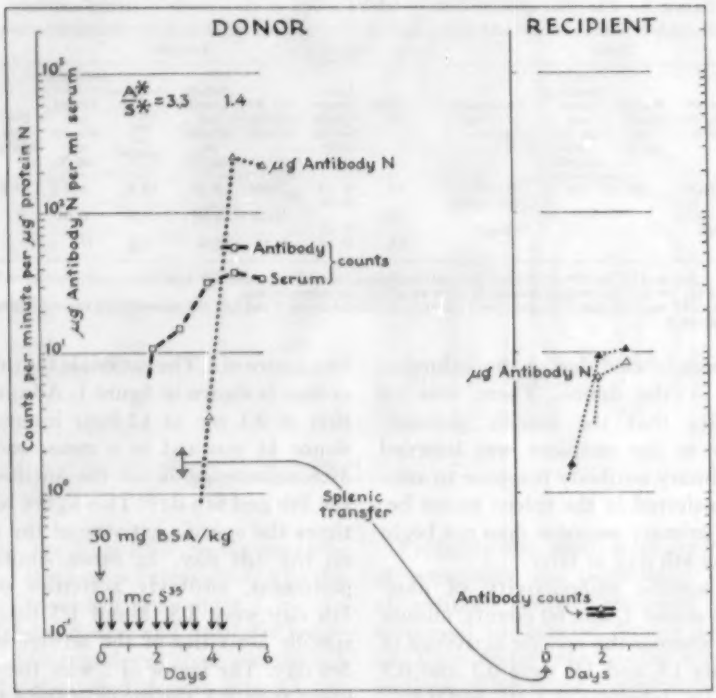


FIG. 2.—A comparison of the antibody formed and its specific radioactivity in labeled donor 14 and its two unlabeled recipients 14A and 14B. In addition, the relation of the specific radioactivity of antibody and serum proteins (A*/S*) are shown in the donor.

activity would be low as compared to the specific antibody label if the latter were being synthesized (cf. the 18-hour interval in fig. 1). As a conservative figure, we estimated the donor antibody as 3 times the serum protein specific activity. The spleen mince from this donor after being washed and injected into recipients 6A and 6B showed an estimated percentage labeling of only about 0.25%. Because of the uncertainty in estimating the specific radioactivity of antibody in donor 6, the spleen mince in donor 14 was similarly washed but the donor was administered S^{35} twice daily for 5 days after receiving antigen so that a direct determination of antibody specific radioactivity could be made (fig. 2). The antibody in recipients 14A and 14B showed approximately the same degree of labeling (0.29%) as was estimated for recipients 6A and 6B. These results substantiate the supposition that coprecipitating and, at times, precipitating antibody is transferred passively in appreciable amounts in unwashed spleen mince.

Therefore, eliminating the 3 highest readings because they probably were augmented by labeled coprecipitating or precipitating antibody transferred passively, the mean labeling of antibody was 0.7% during the induction period as indicated by the data from 6 donors and 10 recipients. The results obtained after washing the spleen mince indicate that 0.3% labeling may even be a truer value.

That a little amino acid incorporation into antibody occurs during the first

3 days† is suggested by the following experiment. The spleen from labeled donor 3 was minced and half of it was injected into recipient 3A. The other half, after mincing and discarding the supernatant, was lysed with distilled water in a Waring blendor and injected into recipient 3B. The spleen of unlabeled donor 8 was also minced and half of it was injected into recipients 3B and 8A. The lysed labeled fraction from donor 3 was transferred to recipient 3B to ascertain whether there was any utilization of labeled amino acids or other precursors of antibody from a spleen containing no living cells. The unlysed unlabeled fraction from donor 8 to recipient 3B was added so that antibody synthesis would occur in the recipient. Had there been any utilization of the radioactive materials from the lysed spleen mince, recipient 3B should have yielded antibody with a specific radioactivity of 1.2 counts/μg N, i.e., the count found in recipient 3A. Its count of 0.01, however, was below the range of experimental significance although it produced antibody commensurate with that produced in recipient 8A. These results, as shown in table 3, indicate that the carry-over of radioactivity in recipient 3A was due to the activity of living cells and not to coprecipitating antibody because the latter would be expected to survive the lysing process and to be passively transferred.

Unlabeled donors and S^{35}-labeled recipients.—Seven recipients received unlabeled spleen mince and were injected with S^{35} during the 2 days of their antibody rise to ascertain whether the resulting antibody was labeled to the same extent as in labeled donor rabbits. The antibody rise in these recipients could thus be compared to the entire immune process when it was completed in one animal where the labeling (1) was limited to a corresponding period, i.e., the 3rd and 4th day, or (2) was given throughout the induction period and antibody rise, i.e., for 5 days.

In comparing the various rabbits, probably the best criterion is the ratio of the specific radioactivity of antibody to that of the serum proteins. This is given in table 4 as A*/S* where A* represents the counts/minute/μg antibody N and S* the counts/minute/μg serum N. These values were determined on the 5th day of antibody rise, i.e., day 2 for all recipients and day 5 for all donors.

The data in table 4 indicate that labeled recipients behaved as if the labeling had taken place in the original immunized animal during the antibody rise and not during the induction period. The following points should be emphasized: (1) The A*/S* ratios varied within a narrow range, 0.9 to 2.5, under several variations in the dosage of S^{35}, i.e., when it was given, how it was given and whether it was given to a recipient or a donor (or a potential donor). (2) The most direct comparison could be made between donor 9 and recipients 9A and 9B. Donor 9 was labeled during its antibody rise in a manner similar to its two recipients, i.e., beginning immediately after splenic transfer. Thus, recipients 9A and 9B (table 4A) produced 8.7 and 4.9 μg antibody N /ml serum, which gave 22.3 and 18.9 counts/minute/μg antibody N. Their A*/S* ratios were 1.4 and 1.8, respectively. Donor 9 (table 4B) formed 856 μg antibody N/ml serum with a specific radioactivity of 17.4 counts/minute/μg antibody N and an A*/S* ratio of 1.3.

† It should be noted that this small amount may not have been actually formed during the induction period. Provided the induction period was shorter than 3 days, the antibody could have been formed during the early part of the antibody rise. Hence, the amount of labeled antibody might have been less had the splenic material been transferred a few hours earlier.

TABLE 4.—*Specific radioactivity of serum and antibody protein N following S^{35}-labeling of recipients and donors. All data were taken on day 2 for recipients and on day 5 for donors.*

Rabbit no.	Mc S^{35} administered	Gram donor spleen per kg recipient	Serum count per minute per μg N	Antibody		A*§ / S*
				μg N /per ml serum	Count per minute per μg N	
	A. Recipients labeled during antibody rise on 0 through 1st day					
7A	1.0:day 1.25	0.95	27.2	20.0	51.3	1.9
8A	0.4:day 0	0.57	18.0	7.1	27.0	1.5
9A	0.1×4	0.39	16.2	8.7	22.3	1.4
9B	0.1×4	0.31	10.6	4.9	18.9	1.8
10A	0.1×4	0.40	8.4	5.9	13.4	1.6
10B	0.4:day 1	0.50	13.9	6.1	24.2	1.7
11A	0.1×4†	0.35	7.3	11.4	18.0	2.5
	B. Donors labeled during antibody rise on 3rd and 4th days					
9	0.1×4		13.4	856.0	17.4	1.3
12	0.2:day 3		8.0	7.6	7.4	0.9
	C. Donors labeled during induction period and antibody rise on 0 through 4th day					
2	0.5×10‡		103.0	2625.0	102.0	1.0
1	0.2×10‡		56.7	987.0	60.0	1.1
11	0.1×10‡		27.0	230.0	40.6	1.5
14	0.1×10		37.5	251.0	53.2	1.4

† The donor for recipient 11A was labeled at this same rate for the 3 days immediately preceding splenic transfer. (It wa also labeled for the 3 following days; see fig. 1.)

‡ Donors 1, 2 and 11 were labeled for an extra day after the values in this table were taken.

§ This ratio $= \frac{\text{antibody count per minute per } \mu g \text{ N}}{\text{serum count per minute per } \mu g \text{ N}}$,

(3) It is interesting that rabbit 12 (injected with BSA a second time as were all the donors but actually not used as a donor) was labeled at approximately the beginning of the antibody rise and showed a low value of 0.9 for A*/S* (table 4B). On the other hand, recipients 7A and 10B were labeled 30 or 24 hours after the splenic transfer and showed a value of 1.9 or 1.7 for A*/S* (table 4A). Since the label probably drops to a very low concentration in the amino acid pool by 12 hours (cf. Dovey et al, 1952), rabbit 12 would be expected to have made less antibody under the influence of labeling and to have had more time for the production of unlabeled antibody than 7A or 10B. Thus, the A*/S* ratio would be expected to be lower in donor 12 than in recipients 7A or 10B. (4) When donors 1 and 2 (table 4C) were labeled through both the induction period and antibody rise, the value for A*/S* was about 1 as contrasted with a mean of 1.6 for recipients only labeled through the antibody rise (recipients 9A, 9B and 10A). This difference is undoubtedly due to the fact that the serum proteins were labeled to a relatively higher degree when the label was given 5 days rather than 2 days and the A*/S* ratio would, therefore, be lower. (5) When recipient 11A and donor 11 were both labeled (the recipient for 2 days during the antibody rise and the donor for 5 days during the induction period and antibody rise), the A*/S* was higher in the recipient (2.5) than in the donor (1.5). Here again, the serum proteins would be expected to have been labeled more and, hence, the A*/S* ratio in the donor would be lower than in the recipient.

The data presented in this section indicate that the incorporation of amino acids into antibody was not perceptibly deranged in the recipient by dividing the immune process between two rabbits.

DISCUSSION

A simple comparison of (1) the low radioactivity of antibody in recipients receiving splenic cells from donors administered S^{35}-labeled amino acids with (2) the high radioactivity of anti-

body in recipients receiving themselves S^{35}-labeled amino acids during the rise of the serum antibody indicates that there is very little incorporation of amino acids into antibody during the induction period. The percent labeled antibody in unlabeled recipients receiving spleen mince from labeled donors given in table 2 indicates that there were the following two groups of animals: (1) 3 rabbits with high values (2A, 2B and 5B) ranging from 3.5 to 5.6% and (2) 10 rabbits with low values ranging from 0.23 to 1.6% (mean of 0.7% labeled). We have already given cogent reasons why the 3 high determinations were too high. Briefly, the passive transfer to recipient 5B of labeled coprecipitating antibody from donor 5 is strongly suggested by determinations on the donor serum at the time of transfer and by the low amount of antibody produced in this recipient as compared to its mate 5A. The passive transfer to recipients 2A and 2B of precipitating antibody is suggested because donor 2 possessed appreciable quantities of precipitating antibody in its serum at the time of splenic transfer. Less than 1% and possibly only 0.3%, therefore, of amino acids was incorporated into antibody during the induction period under the conditions of our experiments. This amount is 3% or less of that reported by Green and Anker (1954).

We do not at present know why our results differ so radically from those of Green and Anker (1954), who concluded that 31% of the antibody was synthesized during the induction period. There may be some difference associated with the different antigens used in the two studies or the immune process may be somewhat deranged by our method of transferring the immune mechanism with spleen mince. More likely it is due to the fact that Green and Anker did not divide the immune process between two animals and did not take into account coprecipitating antibody or complement. These latter materials would have been labeled during the induction period in their work and would have been carried down in the immune precipitate on the 5th day when antibody was isolated for radioassay.

Taken at face value our results indicate that almost all of the sulfur-containing amino acids present in serum antibody are drawn from the amino acid pool during the rise of serum antibody and not during the induction period. The lack of incorporation of amino acids into antibody during the induction period may indicate that synthetic enzymes are developed during the induction period which, once present, begin to form antibody. This in turn suggests the hypothesis of Burnet and Fenner (1949), although all hypotheses of antibody formation necessitate the assumption of new synthetic enzymes either for all or a part of the process of assembling amino acids into the antibody molecule. If very little time elapses between the appearance of the suggested enzymes and the synthesis of antibody, the rise of serum antibody should give an accurate approximation of the rate of antibody formation.

CONCLUSIONS

The secondary immune response to bovine serum albumin was divided between two rabbits by transferring the antibody-forming capacity in splenic materials as follows: Three days after a second injection of antigen into donor rabbits at about the end of the induction period when there was some coprecipitating but usually no precipitating antibody in the serum, the spleens were removed, minced and injected intravenously into normal recipient rabbits. The recipients showed an early rise of

serum-precipitating antibody due to the transferred splenic cells which reached a peak on the second day. Donors or recipients were administered yeast hydrolysate containing amino acids labeled with S^{35}. This procedure led to the following conclusions.

1. Administration to donors of S^{35}-labeled amino acids in single or repeated doses ranging in total radioactivity from 0.4 to 6 mc during the induction period resulted in only a low value of radioactivity of precipitin in recipients. It was estimated that a mean of 0.7% of the total precipitin formed in 10 recipients was labeled during the induction period of 3 days in the donors before the splenic materials were transferred. Moreover, 4 of the 10 recipients which received washed spleen mince gave approximately only 0.3% labeling. Higher values, ranging from 3.5 to 5.6% in 3 recipients, could be partly attributed to the passive transfer of labeled coprecipitating or precipitating antibody from the donors. Some coprecipitating antibody was synthesized during the first 3 days, but has not been adequately studied.

2. The similar administration to recipients (but not to their donors) of S^{35}-labeled amino acids ranging in total radioactivity from 0.3 to 1 mc during the antibody rise resulted in a marked radioactivity of precipitin in the recipients. Specific activities of 1.3 to 2.5 times that of the serum proteins were found. The relative specific activity of serum and antibody proteins was approximately the same in donors and their recipients labeled during the rise of antibody. The incorporation of amino acids into antibody, therefore, was not perceptibly deranged by dividing the immune process between two rabbits.

3. Control passive transfer experiments indicated (1) that S^{35} was stably bound to labeled precipitating antibody in an unlabeled rabbit, and (2) that unlabeled antibody did not acquire an appreciable radioactivity in an S^{35}-labeled rabbit.

4. The results indicate that almost all of the sulfur-containing amino acids present in the precipitating antibody were drawn from the amino acid pool during the rise of serum antibody and not during the first three days after antigen injection (induction period). They also (1) indicate that the rate of rise of serum antibody reflects the rate of antibody formation and (2) suggest that the induction period is concerned with the production of antibody-synthesizing enzymes rather than with amino-acid-containing antibody precursors.

REFERENCES

Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies. Monograph of the Walter and Eliza Hall Institute, Melbourne, Macmillan and Co., Ltd., 142 pp.

Deutsch, L. 1899, Contribution a l'étude de l'origine des anticorps typhiques. Ann. Inst. Pasteur, **13**: 689–727.

Dovey, A., Holloway, R. C. and McFarlane, A. S. 1952, Incorporation of glycine into rabbit plasma proteins. Biochem. J. **51**: XXVI.

Green, H. and Anker, H. S. 1954, On the synthesis of antibody protein. Biochim. et Biophys. Acta, **13**: 365–373.

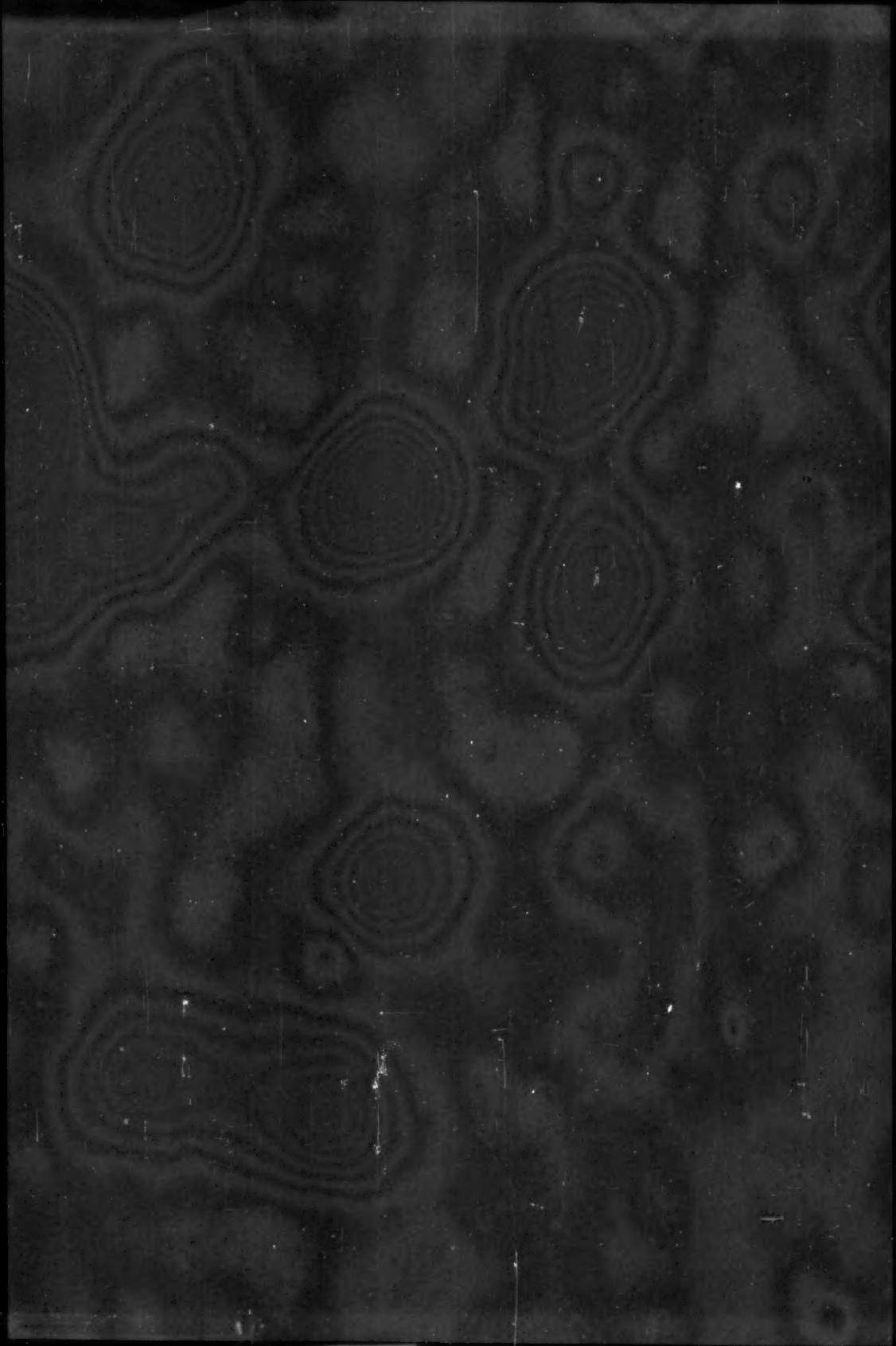
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951, Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

Taliaferro, W. H. and Talmage, D. W. 1955, Absence of amino acid incorporation into antibody during the induction period. Science, **121**: 626.

Talmage, D. W., Baker, H. R. and Akeson, W. 1954, The separation and analysis of labelled antibodies. J. Infect. Dis. **94**: 199–212.

Talmage, D. W., Dixon, F. J., Bukantz, S. C. and Dammin, G. J. 1951, Antigen elimination from the blood as an early manifestation of the immune response. J. Immunol. **67**: 243–255.

Talmage, D. W. and Maurer, P. H. 1953, I^{131}-labelled antigen precipitation as a measure of quantity and quality of antibody. J. Infect. Dis. **92**: 288–300.



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